UNIVERSITY OF LONDON THESIS

Degree PhD Year 2008 Name of Author SLOAN, Richard David

STUDIES INTO THE TRANSMISSION OF HEPATITIS B VIRUS THAT MUTATE FOLLOWING THERAPY WITH NUCLEOSIDE ANALOGUES AND THE POTENTIAL FOR SUCH TRANSMISSION TO BE SEROLOGICALLY UNDETECTABLE

A thesis submitted for the award of Doctor of Philosophy

By

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April 2007

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ACKNOWLEDGEMENTS

I would like to thank those who supervised this project; both Dr Chong Gee Teo and Professor Richard Tedder provided me with the opportunity to begin this work, and gave me the independence to direct my own research and learn from my own mistakes. Dr Samreen Ijaz especially was a source of continual support and I have benefited from numerous inspirational discussions with her. All three must be thanked for the critical reading of this manuscript.

I was fortunate enough to have undertaken these studies at no less than three excellent scientific institutions; the Centre for Infections of the Health Protection Agency, The Royal Free and University College Medical School and the Department of Virology University College London. I must too thank the many staff who gave so many forms of assistance.

Specifically mention must be given Dr Tim Harrison and Dr Penny Moore who both aided me with the construction of expression vectors, as well as providing me with cell lines, training and from whom I had many valuable suggestions. Additionally Dr Nikolai Naoumov and Dr Sam Coward are thanked for also providing me with cell lines. I am hugely grateful to Dr Julian Copeland of Abbot-Murex who donated all the ELISA regents which were the essential component of these studies.

My family who have been so supportive of this endeavor, are to be thanked, especially my parents (and patrons) who has always been so encouraging. Finally the greatest debt of thanks is owed to my partner Susie whose unflagging support and confidence in this work has given me much needed emotional support throughout.

This work was funded by a grant provided the European Community 5th framework program.

Abstract: The hepatitis B virus (HBV) genome is organised so that the open reading frame encoding the polymerase overlaps that encoding hepatitis B virus surface antigen (HBsAg). Previous studies showed that acquisition of lamivudine resistance-associated mutations in *pol* result in a decreased affinity of HBsAg for its antibody (anti-HBs). It was sought to characterize epitope changes in the major immunogenic domain of HBsAg, the "a" determinant, that result from lamivudine, adefovir and entecavir resistance mutations. Recombinant (r) HBsAg was produced by transfecting Chinese Hamster Ovary cells (CHO) with a plasmid containing the surface open reading frame modified by site-directed mutagenesis to mimic mutations selected in the overlapping *pol*/S gene during antiviral therapy. Wild type and mutant rHBsAgs expressed from these constructs were assayed using a series of EIAs each employing a monoclonal antibody which binds distinct epitopes in the first and second loop of the "a" determinant. Specific combinations of mutations led to variable loss of immunoreactivity of the epitopes of the "a" domain, despite some mutations not being located in that domain. Some combinations of mutations led to restoration of reactivity of epitopes which were abrogated by single mutations. Thus mutations associated with antiviral resistance have the potential to affect serological reactivity through concomitant amino acid substitutions in HBsAg. These observations may have implications for the clinical treatment of chronic HBV, diagnostics and vaccine programmes. The molecular epidemiology of antiviral-associated mutants in England and Wales between 1997-2001 was also assessed. HBV DNA from the 1st 600 bases of the HBsAg-coding gene was amplified from 163 patients with acute hepatitis B and subjected to phylogenetic analysis. None of the patients were found infected with HBV mutants that would have arisen following the development of antiviral resistance or with vaccine-escape mutants. Transmission by pre-existing iatrogenic HBV mutants was therefore rare or did not occur.

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ABBREVIATIONS

aa	amino acid
ALT	alanine aminotransferase
AUC	area under curve
bp	base pairs
cccDNA	covalently closed circular DNA
cDNA	complementary DNA
CHB	chronic hepatitis B
ddH_2O	double distilled water
DHBV	duck hepatitis B virus
DMEM	Dulbecco's Minimal Essential Medium
DMSO	dimethyl sulphoxide
DNA	deoxyrobonucleic acid
dsDNA	double stranded DNA
ER	endoplasmic reticulum
ELISA	enzyme linked immune absorbent assay
g	grams
HAV	hepatitis A virus
HBcAg	HBV core antigen
HBeAg	HBV e antigen
HBIG	HBV immune globulin
HBs	HBV surface protein
HBsAg	HBV surface antigen
HBV	hepatitis B virus
HBx	HBV X protein

HCC	hepatocellular carcinoma
HDV	hepatitis D virus
HCV	hepatitis C virus
HEV	hepatitis E virus
HFV	hepatitis F virus
HGV	hepatitis G virus
HIV	human immunodeficiency virus
IDU	injecting drug user
IFN	interferon
Kb	kilobases
kDA	kilo-Daltons
LB	Luria-Bertani broth
LHBs	large HBV envelope protein
M	Molar
MAb	monoclonal antibody
MHBs	medium HBV envelope protein
MHR	major hydrophilic region
ml	milliliter
mg	milligram
MSM	men who have sex with men
Nm	nanometer
NANBH	non-A non-B hepatitis
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMV	PM virus

pI	isoelectric point
pgRNA	pregenomic RNA
rcDNA	relaxed circular DNA
RNA	ribonucleic acid
RT	reverse transcriptase
SHBs	small HBV envelope protein
SNP	single nucleotide polymorphism
TBS	Tris buffered saline
TTV	TT virus
TBST	Tris buffered saline containing 0.02% tween 20
µl	microlitre
µM	micro molar
WHO	World Health Organization
WHBV	woodchuck hepatitis virus

AMINO ACID ABBREVIATIONS

A	Ala	alanine
C	Cys	cysteine
D	Asp	aspartic acid
E	Glu	glutamic acid
F	Phe	phenylalanine
G	Gly	glycine
H	His	histidine
I	Ile	isoleucine
K	Lys	lysine
L	Leu	leucine
M	Met	methionine
N	Asn	asparagine
P	Pro	praline
Q	Gln	glutamine
R	Arg	arginine
S	Ser	serine
T	Thr	threonine
V	Val	valine
W	Trp	tryptophan
Y	Tyr	tyrosine

CHAPTER 1

GENERAL INTRODUCTION

1.1: The discovery of hepatitis B virus

Hepatitis, i.e. inflammation of the liver, has been recognised for centuries owing to its most recognisable symptom, jaundice, a yellow-orange skin discolouration now known to be caused by the accumulation of bile pigment following reduced excretion by damaged hepatocytes. Outbreaks of jaundice were commonly reported during military campaigns from the 17th to early 20th centuries, when the disease became known as "campaign jaundice" (Zuckerman, 1979). Outbreaks of jaundice were also common amongst civilians. In 1912, Cockayne named such jaundice epidemics as "infective hepatitis". In the late 1930s and early 1940s it became clear that hepatitis could be parenteraly transmitted. This was particularly evident upon the mass immunisation of Second World War troops against diseases such as mumps and yellow fever using vaccines containing human serum. This second form of hepatitis was termed "serum hepatitis". In 1967, Saul Krugman identified that the epidemiology of hepatitis outbreaks pointed towards two distinct modes of disease: one transmitted by the faeco-oral route and the other parenteral. He named these hepatitis A and hepatitis B, respectively (Krugman *et al.*, 1967). Whilst looking for polymorphic serum proteins from humans in different geographic areas, Baruch Blumberg serendipitously discovered the "Australia Antigen" in the blood of an Australian Aborigine. This was a serum antigen that reacted against antibodies derived from multiply transfused haemophilia patients (Blumberg *et al.*, 1965). This antigen, was initially thought to be a glycoprotein phenotype associated with leukaemia, but was later clarified to be associated with hepatitis B (Blumberg *et al.*, 1967, Prince, 1968) and is now known as hepatitis B virus surface antigen (HBsAg). In 1970, DS Dane, using direct electron microscopy, identified 42-nm virus-like particles, which bore Australia Antigen on the surface (Dane *et al.*, 1970). These particles, which are virions of hepatitis B virus

(HBV), acquired their discoverer's name and are still known as "Dane particles". Dane particles were found to contain endogenous polymerase activity within the core (Kaplan *et al.*, 1973), which was further indicative of the viral nature of Dane particles, and confirmed to be so by the eventual characterisation of the HBV genome (Robinson *et al.*, 1974).

The development and implementation of laboratory testing for HBV has identified that the virus can be transmitted sexually, perinatally, percutaneously as well as parenterally, and that infection, which can be icteric or asymptomatic, can lead to chronic infection in adults occasionally, but more often in neonates and infants. Such knowledge of the routes of transmission and the clinical progression of infection has allowed HBV infection to be distinguished from other causes of viral hepatitis.

1.2: Other causes of viral hepatitis

1.2.1: Hepatitis A virus

Hepatitis A virus (HAV) was discovered in the stools and livers of infected patients (Feinstone *et al.*, 1973). This piconarvirus is transmitted by the faeco-oral route and causes and acute self-limiting disease. A vaccine has been developed (Gust, 1990) which is efficacious in preventing pre and post-exposure hepatitis A (Gust *et al.*, 1988).

1.2.2: Hepatitis C virus

Following the wide availability of laboratory testing for HAV and HBV, it became evident that a substantial proportion of post-transfusion hepatitis and jaundice cases among injecting drug users was not due to HAV and HBV; this entity was termed non-

A non-B hepatitis [NANBH], (Feinstone *et al.*, 1975; Dienstag *et al.*, 1983). In 1989, a report was made of the discovery of a virus identified in the blood of NANBH-infected chimpanzees; this was hepatitis C virus (HCV) (Choo *et al.*, 1989). The HCV genome was eventually characterised and HCV shown to be related to the flaviruses (Miller *et al.*, 1990). It was then demonstrated that HCV was the major cause of NANBH and infection by this virus was extensive in multiply transfused haemophiliacs. The screening of blood for antibody to HCV has greatly reduced the incidence of NANBH. While HCV is transmitted parenterally and perinatally, sexual transmission is rare. The large majority of people infected by HCV develop chronic infection, which can be asymptomatic but can lead to end-stage liver disease and hepatocellular carcinoma. The disease can be treated with interferon-alpha (IFN-α) with varying degrees of success (Terrault, 2005). There is currently no vaccine available.

1.2.3: Hepatitis D Virus

The hepatitis D virus (HDV) was discovered by in 1977 upon the identification through direct immunofluoresence of the novel "delta antigen" expressed in HBV infected hepatocytes (Rizzetto *et al.*, 1977). HDV is now known to be a defective virus, reliant on HBV infection for virion production to occur as it requires HBsAg as its coat. Infection with HDV can aggravate some chronic HBV infections leading to a more severe disease course. Though there is currently no available vaccine, the extent of HDV infection can be limited by immunisation against HBV (Koff, 2003).

1.2.4: Hepatitis E Virus

Hepatitis E Virus (HEV) was discovered upon the investigations of an outbreak of enterically transmitted NANBH in India (Khuroo, 1991). Although it was considered the outbreak may have been due to HAV, the agent responsible was shown to have distinct antigenic properties. Calcivirus-like particles were identified in the stools of infected individuals (Balayan *et al.*, 1983) and Koch's postulates were fulfilled following challenge experiments in macaques (Krawczynski *et al.*, 1989). Hepatitis E virus infection is particularly dangerous in pregnant women, in whom acute infection can lead to fulminant disease route. There are now a number of serological and molecular assays available for HEV (Humphrey *et al.*, 1990; Lau *et al.*, 1991).

1.2.5: Non A-E hepatitis viruses and other agents

Although there are now diagnostic assays for the detection of the viruses outlined above, cryptogenic cases of hepatitis of presumed viral cause still occur (Alter, 1994; Thiers *et al.*, 1993) and novel candidate viruses are being proposed.

Hepatitis F Virus

One candidate virus, named hepatitis F virus (HFV), has been proposed as the cause of hepatitis in a small number of human cases. Intravenous transmission of stool concentrates could be demonstrated in rhesus monkeys which went on to develop hepatic lesions. The importance of HFV as a clinically important infection of humans remains unclear (Deka *et al.*, 1994).

Hepatitis G Virus (GBVC)

Hepatitis G virus was discovered by cloning the virus, using the polymerase chain reaction [PCR] (Leary *et al.*, 1995; Simons *et al.*, 1995). The epidemiology of the virus

is far from clearly understood though transmission through transfusion has been demonstrated (Schmidt *et al.*, 1996). There is, however, no evidence that this virus produces symptomatic disease and it now seems probable that it does not cause viral hepatitis at all.

TTV

TT Virus is named after the initials (TT) of the index case. It was discovered in Japan in following study of patients with fulminant and chronic hepatitis of unknown aetiology (Nishizawa *et al.*, 1997). The virus may be found in 25% of patients with chronic liver disease (Naoumov *et al.*, 1998). Despite such associations there is no direct evidence that TTV causes symptomatic disease upon infection and it has been suggested that TTV, like HGV, may not cause viral hepatitis (Naoumov *et al.*, 1998).

PM Virus

A TTV like, but highly genetically divergent virus was identified from a single patient with acute non A-E hepatitis (Hallet *et al.*, 2000). Owing to its extreme genetic divergence from TTV, this virus, PMV, may be the prototype of an independent taxonomic group within the *Circoviridae* family. Typically circoviruses are porcine pathogens and so their description as a human infection is unusual. A geno-prevalence study suggested infection by this virus to be rare. Like HGV/GBVC and TTV, there is no firm evidence that this virus is a significant cause of viral hepatitis.

Other viruses such as mumps virus, yellow fever virus, rubella virus, Epstein Barr Virus and cytomegalovirus can also cause hepatitis, but more often within the context of a larger constellation of disease manifestations.

1.3: Clinical features of HBV infection

Acute HBV infection can be sub clinical (sometimes known as *inapparent* hepatitis) or icteric. Most HBV infections are sub clinical, especially during childhood, but about one third of adult infections are icteric. The vast majority of HBV infections acquired in adulthood result in a self limiting disease. Acute hepatitis B can proceed along 4 clinical phases: the incubation period, the preicteric stage, the icteric phase and the convalescent period. The incubation period is typically 45-120 days, and incubation periods of less than 45 days or more than 150 days are considered unusual. Following the incubation period the preicteric stage is characterised by mild fever, lethargy, malaise, anorexia, myalgia, nausea and vomiting. Patients such as older children and adults may also report right upper quadrant pain due to hepatomegaly (Ward *et al.*, 1958). The appearance of dark, golden-brown urine marks the beginning of the icteric phase; this is followed around one or two days later by pale stools and jaundice. Generally, the icteric phase subsides after between two to six weeks and the patient enters the convalescent phase whereupon the damaged liver is regenerated.

Occasionally, during acute viral hepatitis, more extensive necrosis of the liver occurs that leads to severe impairment of hepatic synthetic processes, excretory functions, and detoxifying mechanisms. If this occurs during the first 8 weeks of illness, it is designated as fulminant hepatitis and is characterised by the sudden onset of high fever, marked abdominal pain, vomiting and jaundice, followed by development of hepatic encephalopathy with deep coma seizures (Adams *et al.*, 1953, Berk *et al.*, 1978). In 70%-90% of patients, ascites, a bleeding diathesis, renal dysfunction and decerebrate rigidity may develop which can lead to death.

A small proportion of adulthood infections (1-5%) and a large proportion (90%) of infections acquired perinatally, in the neonatal period or early childhood progress to chronic hepatitis (Hyams, 1995). In older children, the risk of chronicity decreases to about 30%. Chronic hepatitis B can be defined as HBs antigenemia persisting for at least 6 months. Chronic infection may be asymptomatic, such carriers typically show absent or mild histological and biochemical evidence of liver injury. Chronic persistent or chronic active disease can also occur, which may cause progressive liver damage that may lead to cirrhosis and/or to hepatocellular carcinoma (HCC). The rapid course of malignancy and the poor prognosis of HCC results in an annual mortality rate from HCC that is virtually the same as its annual incidence (Evans and London, 1998).

1.4: Epidemiology and Transmission of HBV

1.4.1: Worldwide epidemiology

The World Health Organisation (WHO) estimates that three billion people worldwide (i.e., about one third of the world's population) have been infected with the hepatitis B virus, and of these more than 300-400 million are chronic carriers of the virus (Kane *et al.*, 1995; van Damme *et al.*, 1995; Lee *et al.*, 1997). More than one quarter of people chronically infected with HBV will die of associated HCC (Beasley *et al.*, 1988). Worldwide deaths from HBV-induced liver cancer probably exceed one million per year (Evans and London, 1998; Parkin *et al.*, 1999). The worldwide endemicity of HBV infection is presented in figure 1.1 below.

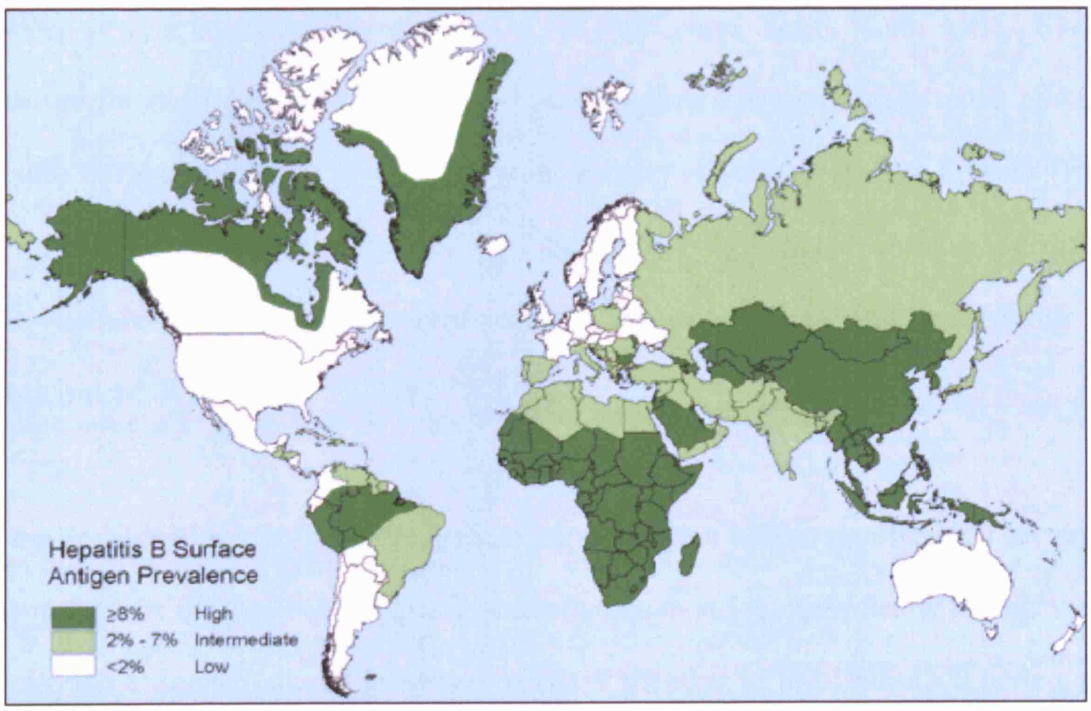


Figure 1.1 – Global endemicity of HBV infection (source: www.cdc.gov/hepatitisb).

In the developing world, 8-15% of individuals are chronic carriers. Some 5-12% of parturient women are HBsAg-positive, of whom about half are HBV DNA positive: when perinatal transmission occurs where the mother is HBsAg positive and without vaccination cover, there is a 70-90% chance that the infant will become infected. For such infected infants there is an 85% chance that they will become a carrier.

In South Africa, between 5%-10% of pregnant women are estimated to be in the highly infectious HBeAg positive carrier state (Prozesky *et al.*, 1983; Guidozzi *et al.*, 1992), while in South East Asia more than 40% of pregnant women are HBeAg-positive (Edmumds *et al.*, 1996). As a consequence, East Asian babies are more commonly infected at birth (Stevens *et al.*, 1975 and 1979). The majority (85%) of those born to HBeAg positive mothers become persistently infected (Beasley *et al.*, 1977), while only 30% of those born to HBeAg negative mothers do so, this is likely due to the role of HBeAg as an immune system toleragen in HBV infection. Yet in South Africa, though endemic for the infection, vertical transmission appears to be rare and most children acquire HBV horizontally, at ages 1-5 years through as yet undetermined means (Hino *et al.*, 2001). Because the development of chronicity is age-related, childhood acquired horizontal infections and perinatally acquired infections impose the greatest risk for development of carriers.

In the developed world, however, perinatal transmission is less significant. The carrier rate in females of child bearing age is generally less than 1%. Specifically in England & Wales, the estimated annual incidence of HBV infection in the 1995-2000 period was 7.4 per 100,000 (Hahne *et al.*, 2004). Transmission is principally horizontal, not vertical (Ramsay *et al* 1998, Kane *et al* 1993). The primary routes of horizontal transmission is sexual (including men who have sex with men [MSM]) and parenteral transmission,

with injecting drug users (IDUs) being the most commonly reported risk of transmission, reported in 53% of males and 43% of females in England & Wales (Hahne *et al.*, 2004). The routine testing of blood, plasma and blood products for HBsAg has ensured that the risk of post-transfusion hepatitis has almost disappeared. However, HBV infection is still considered a risk to unimmunised health care and laboratory workers, close contacts of chronically infected patients and the patients of haemodialysis units. Due to low incidence and prevention strategies for both sexual, parenteral and perinatal transmission outbreaks are rare and there is no seasonal trend for HBV.

1.4.2: Perinatal transmission

In certain areas of the developing world, perinatal transmission is the most important mechanism for maintenance of an HBV carrier population. It has been estimated that at least 23% of the carriers in Asia and 8% in Africa result from perinatal transmission (Kane *et al.*, 1993).

Infected infants are usually HBsAg-negative at birth but develop HBs antigenemia at 1-3 months of age. Although HBsAg can be detected in cord blood (Stevens *et al.*, 1975), its presence does not correlate with the detection of HBsAg in the infant's circulation at birth (Lee *et al.*, 1978; Stevens *et al.*, 1975). HBsAg found in the cord blood may also be due to contamination with maternal HBsAg, whereas HBsAg in the infant may also be due to HBV having crossed the placental barrier (Lee *et al.*, 1978).

In Africa horizontal transmission during childhood is the usual route of infection that leads to chronic carriage, children are thought to be infected later in life possibly by saliva or maternal milk (Botha *et al.*, 1984; Hino *et al.*, 1991). Transmission of HBV between familial contacts is relatively common through highly infectious HBeAg

positive carriers and blood is the suspected route of transmission (Szmuness *et al.*, 1970) perhaps through scratches, insect bites, skin mites and sores. This contrasts to Asia, where the usual route of infection that leads to chronic carriage is perinatal transmission.

1.4.3: Parenteral Transmission

The introduction of parenteral therapies, mass immunisation campaigns and the extensive transfusion of blood products permitted the recognition of hepatitis B as a major health problem (Mosley, 1965). The propensity of HBV to induce long incubation periods, asymptomatic infections and an infectious carrier state make HBV well suited to transmission by blood. The risk of such transmission occurring has, however, been decreased by the screening of blood for HBsAg, the use of volunteer blood donor population and the development of virucidal techniques to render blood components (such as red blood cells, plasma and platelets) and products (such as Factor VIII and Factor IX) safe.

Some risk groups are still susceptible nonetheless to parenteral infection, particularly haemodialysis patients. The stability of HBV and its retention on mechanical surfaces, including those involved in dialysis partially explains the susceptibility of patients undergoing haemodialysis to HBV infection. This stability underlies the high rate of incidence observed in injecting drug users, which is associated with the sharing of contaminated needles, syringes and other paraphernalia. Healthcare workers are also at risk from parenteral HBV infection acquired through needle stick injuries and occupational exposure to blood and other body fluids (Maynard, 1978).

1.4.4: Non-sexual, non-parenteral transmission

It is probable that non-parenteral, non-sexual transmission of HBV may occur, although there is little evidence for it. As well as occurring in serum, HBsAg and HBV DNA have been demonstrated in a variety of body fluids including sweat, breast milk, pleural fluid, vaginal secretions, saliva, semen, urine and faeces (Doblin *et al.*, 1985; Davison *et al.*, 1987). The presence of HBV in body secretions may allow non-parenteral transmission to occur, and such transmission may in part explain the infection of close contacts of chronic HBeAg-seropositive carriers (Szmuness *et al.*, 1970). The presence of HBV in body secretions has been also used to explain horizontal transmission amongst African infants (Botha *et al.*, 1984; Hino *et al.*, 1991) though parenteral transmission through other routes is more likely.

1.4.5: Sexual Transmission

There are strong epidemiological associations between the sexual behaviour of certain patient groups and HBV infection. One early study detected HBsAg in the sera of 15 of 658 men and in 2 of 316 women attending a sexually transmitted disease clinic in London (Fulford *et al.*, 1973). Anti-HBs was also detected in 36 men and 11 women of the same group, indicating previous infection. The study noted that there was also a significant correlation between the presence of HBsAg and the patients' country of origin, particularly those from Middle Eastern or Mediterranean countries. A significantly greater proportion of patients with three or more sexual contacts had serum anti-HBs. A later study from the same investigating group showed anti-HBc in 48% of homosexual men tested, indicative of current or previous HBV infection (Gilson *et al.*, 1990). In contrast, in heterosexual men the rate of anti-HBc was 7.5%. Other studies revealing virus in semen and asymptomatic mucosal lesions (Reiner *et al.*, 1984) as well

as vaginal secretions (Doblin *et al.*, 1985; Davison *et al.*, 1987) indicated a potential mechanism for the transmission of HBV.

1.5: The structure of HBV

1.5.1: Virus morphology

Within the blood of an infected person may be found 3 types of HBV particles with differing morphologies. The 42-nm Dane particle is the "true" (i.e. infectious) viral particle. The virus is a spherical double-shelled structure – the outer shell is comprised of HBsAg protein (in 3 forms – small [S], medium [M] and long [L]), with the inner shell, referred to as the core particle or capsid, being comprised of HBc protein. The core encloses the partially double stranded HBV DNA genome to which the viral polymerase is covalently attached at tyrosine 63 through a phosphodiester bridge to the 5' end of the L(-) strand (Weber *et al.*, 1994). There are also a number of other host proteins that associate with the virus-encoded structural proteins. These include a cellular protein kinase C within the core particles (Gerlich *et al.*, 1992), which is responsible for phosphorylation of serine amino acids within the carboxyterminal portion of the core protein (Kann *et al.*, 1993).

A heat shock protein, hsc70, associates with the internal preS1 domain of duck HBV (DHBV) and a number of other proteins are thought to associate more loosely with the envelope of the virus, including polymerised human serum albumin (pHSA) and anti-HBs antibodies (Kann and Gerlich, 2000). There are also two types of subviral particles, a spherical 22 nm particle with octahedral symmetry (Gilbert *et al.*, 2005) comprised of HBs protein called small forms/particles (these particles are extremely abundant

reaching concentrations of 10^{11} per ml in the serum) and filamentous particles of approximately 20 nm in diameter and of variable length (also comprised of HBs but less abundant in serum (Dane *et al.*, 1970) [see figure 1.2]. Neither subviral particle contains DNA and so are not infectious. A depiction of the virion structure is shown in figure 1.3.

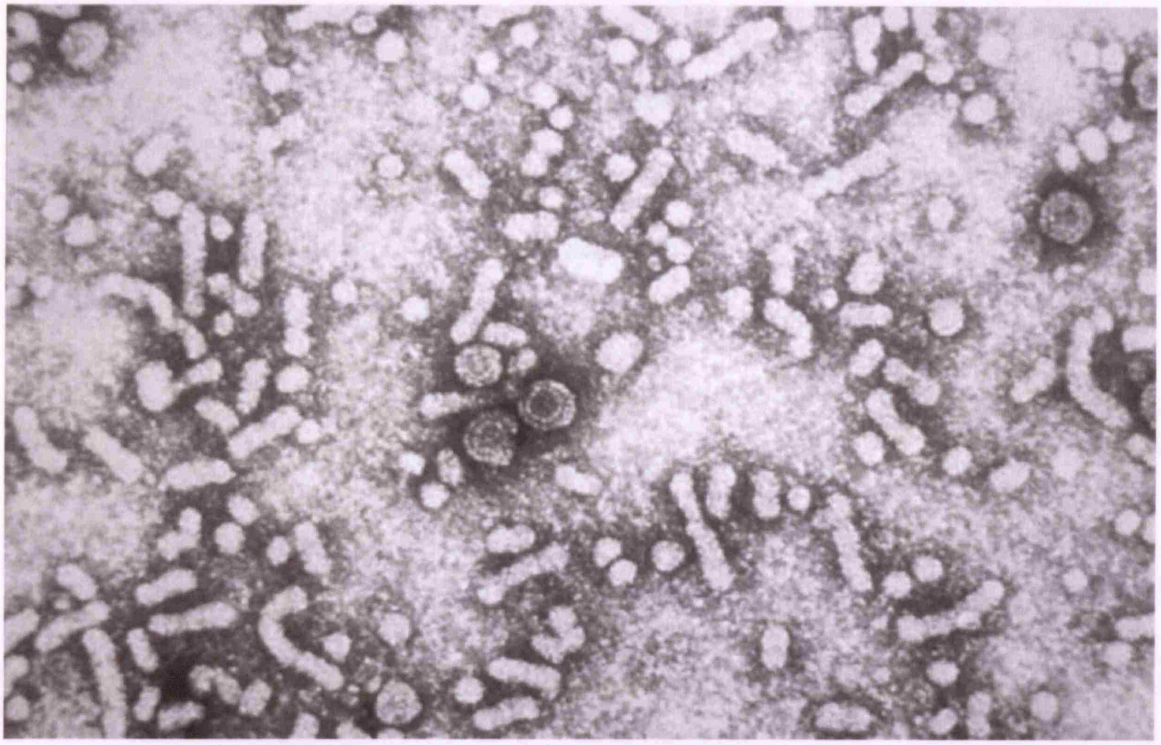


Figure 1.2 - Electron micrograph showing the Dane particle and both the filamentous and spherical subviral particles (source: www.prn.org/hepb785.jpg)

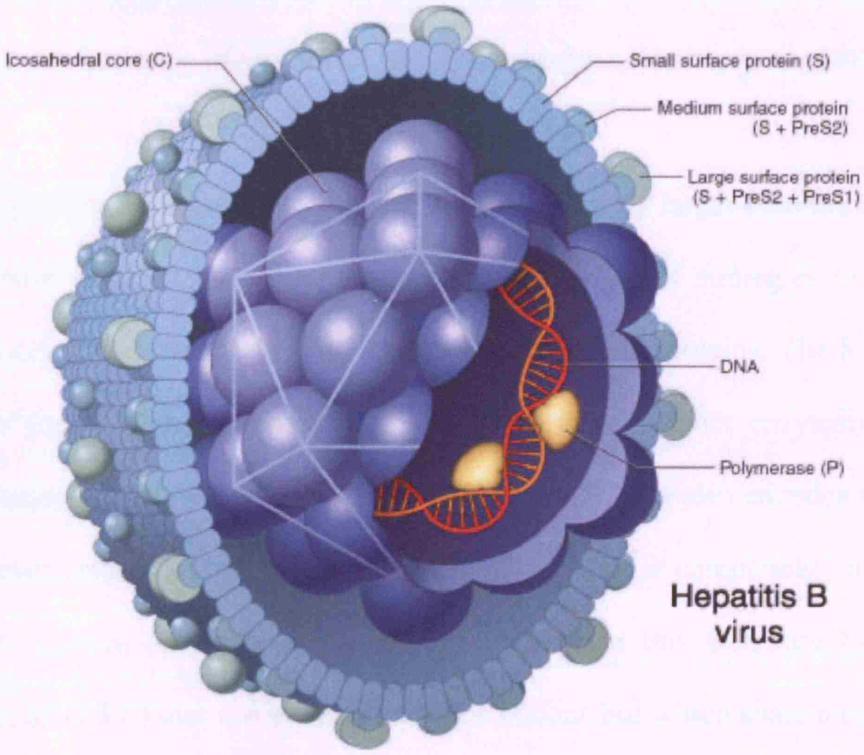


Figure 1.3 – Schematic showing structure of hepatitis B virion

(source:http://www.rit.edu/~japfaa/HBV.jpg)

1.5.2: HBV Genome Structure

The genome of the hepatitis B virus is a circular, partially double-stranded DNA molecule (Robinson *et al.*, 1974) with a single stranded region of variable length. The longer stand, the minus or L (-) DNA strand, which is complementary to the mRNAs, is approximately 3.2 kb in length. Conventionally, numbering of the bases within the genome starts at the *EcoR1* cleavage site within the genome (or at the corresponding position in those variants which do not encode that restriction site) (Kann and Gerlich, 2000). The shorter plus or S(+) strand is approximately 50-70% of the length of the L(-) strand, with the position of the 5' ends of both strands being fixed and the position of the 3' end of the S(+) strand being variable. The partially single-stranded nature of the genome results in a relaxed circular conformation (rcDNA). The circular conformation of the viral genome is maintained by the presence of 224 bp 5' cohesive termini, which also contain an 11-bp direct repeat (DR1 and DR2, 5' TTCACCTCTGC) that is required for the initiation of viral DNA synthesis (Molnar-Kimber *et al.*, 1984).

The HBV genome is remarkably compact. It is only slightly larger than the largest open reading frame (ORF) of the virus and utilises a number of strategies to encode for separate ORFs which themselves may encode for multiple proteins. The longest ORF, P, encodes the viral polymerase, which performs three distinct enzymatic functions (DNA polymerase, reverse transcriptase and RNAase H) and also encodes the terminal protein primer (Bosch *et al.*, 1988). The preS1/S2/S ORF is completely located within ORF P but utilises an alternative reading frame. From this ORF are transcribed 3 proteins (S, M and L) that use internal initiation codons but which share a common stop codon. These proteins encode for the viral envelope protein (Valenzuela *et al.*, 1979). ORF C encodes the HBc and HBe proteins through the use of two initiation sites but a common stop codon (Pasek *et al.*, 1978) and is partially overlapped by ORF P. ORF X,

also partially overlaps ORF P and encodes the HBx protein which is believed to be a transactivating protein that upregulates transcription from all viral and some cellular promoters (Rossner, 1992) Regulatory genetic elements which control the viral transcription, RNA processing and translation of proteins are also situated within coding regions (Kann and Gerlich, 2000). There are two additional, potential open reading frames for which no translational products have been identified (Miller *et al.*, 1989). The structure of the HBV genome is represented in figure 1.4 below.

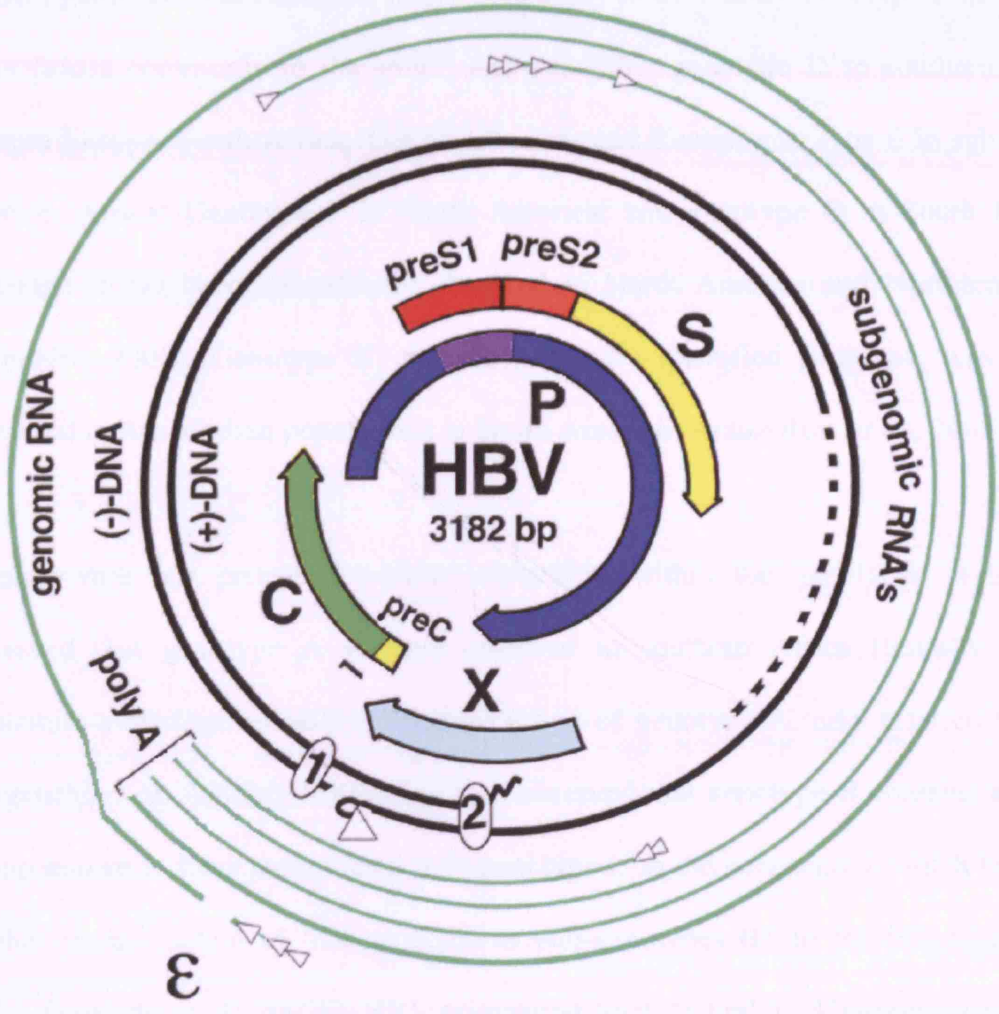


Figure 1.4 – The genomic structure of HBV. (Source: http://www.molecular-virology.uni-hd.de/rsr/hbv/1_morphology_and_genomeorganization.htm).

1.6: HBV genotypes

HBV can be grouped into 8 genotypes (A-H), which differ from each other by more than 8% at the nucleotide level of the complete genome sequence (Okamoto *et al.*, 1988) or 4% of the S-gene sequence (Norder *et al.*, 1992). There exists partial correlation between the serological subtypes of HBV and HBV genotype (Norder *et al.*, 1992; Ohba *et al.*, 1995). The genotypes show a distinct geographic distribution. Genotype A is found predominantly in northern Europe, North America and southern Africa; genotype B in East Asia (more commonly to the North); genotype C in also East Asia (more commonly to the south) and Australia; genotype D in southern Europe, eastern Europe, North Africa, The Middle East and Russia; genotype E in sub-Saharan western Africa; Genotype F in South America; and genotype G in South America, although it has been sporadically observed in North America and Northern Europe (Schaefer, 2005). Genotype H, the most recently identified genotype, was initially observed in Amerindian populations in South America (Arauz-Ruiz *et al.*, 2002).

Recent work has proposed various sub-groups within the genotypes, it has been observed that genotype A variants observed in southern Africa (initially Malawi) constitute a phylogenetically distinct subgroup of genotype A, now referred to as Aa (Sugauchi *et al.*, 2003a). It has also been observed that genotype B contains a distinct group known to have recombined with genotype C in the core region, which had led to further categorisation of this genotype as sub-genotypes B1 to B4 (Sugauchi *et al.*, 2002, Sugauchi *et al.*, 2003b). HBV originating from Australian Aborigines constitute a distinct genotype C sub-genotype, was briefly as designated $C_{australia}$ (Sugauchi *et al.*, 2001) though the genotype has now been further subdivided. It has been observed, from whole genome phylogenetic analysis, that genotype D can be split into 4

phylogenetically distinct groups D1-D4 (Norder *et al.*, 2004). Genotype F can also be split into two subgroups, F1 and F2 (Norder *et al.*, 2003). With the increase in availability and the decreasing prices of DNA sequencing, the discovery of new sub-genotypes becomes increasingly likely. What is clear is that there is currently no common method for sub-genotype nomenclature, nor even agreed nomenclature for sub groups within genotypes, with some groups even using the term subtypes, which is easily confused as serologically determined subtypes. As was determined for HCV (Simmonds *et al.*, 2005), a common nomenclature system is necessary, that proposed by Norder and colleagues is most apt.

There is growing appreciation that there are clinical differences associated between HBV genotypes (Wai & Fontana, 2004; Schafer, 2005). Though most work on genotype differences originates from East Asia and these related to differences between genotypes B and C, data showing genotype-specific biological differences are far from comprehensive, especially those relating to the common European genotypes (A and D). Nonetheless, there is gathering evidence for genotype-associated differences. Genotype A more commonly associated with chronicity of infection than genotypes B or C (Kikuchi *et al.*, 2000). Genotype C has been reported to be associated with greater severity of chronic infection than genotype B and higher incidence of cirrhosis (Kao *et al.*, 2000); Patients infected with genotype C appear to more likely progress to HCC than those infected with genotype B (Tsubota *et al.*, 2001). HBeAg-seronegative chronic infection may also be affected by genotype, being more likely in genotype B than genotype C infections (Orito *et al.*, 2001). Serological subtypes, which roughly correlate with genotypes, have also been shown to play a role in the immunological response to pre-S2 antigens. Therefore some subtypes are associated with poorer anti-HBs response (*adr*, *ady*) (Milich *et al.*, 1990a, Milich *et al.*, 1990b). It was also

demonstrated that genotype C is associated with an increased likelihood of vaccine escape mutants being selected in vaccinated children born to HBV DNA positive mothers (Ngui *et al.*, 1998). Moreover, genotype B is associated with a better response to interferon therapy compared to genotype C (Kao *et al.*, 2000, Wai *et al.*, 2002, Janssen *et al.*, 2005). In a similar comparison between genotypes A and D, patients with genotype D responded better (Hou *et al.*, 2001), whilst genotype A showed a very low response rate compared to genotypes B and C (Kobayashi *et al.*, 2002). Genotype may also play a role in the rate at which antiviral resistance emerges; while in the long term (2 years) the likelihood of lamivudine resistance emerging is equal for all genotypes, for patients infected with genotype D lamivudine resistance tends to arise more rapidly (Buti *et al.*, 2002; Zollner *et al.*, 2002). Finally, it has also been noted that infection with genotypes A or D tends to lead to differing patterns of lamivudine resistance mutations at the YMDD motif of HBV reverse transcriptase (Hadziyannis *et al.*, 2000). Given these findings genotype determination may be useful in devising patient management and public health strategies.

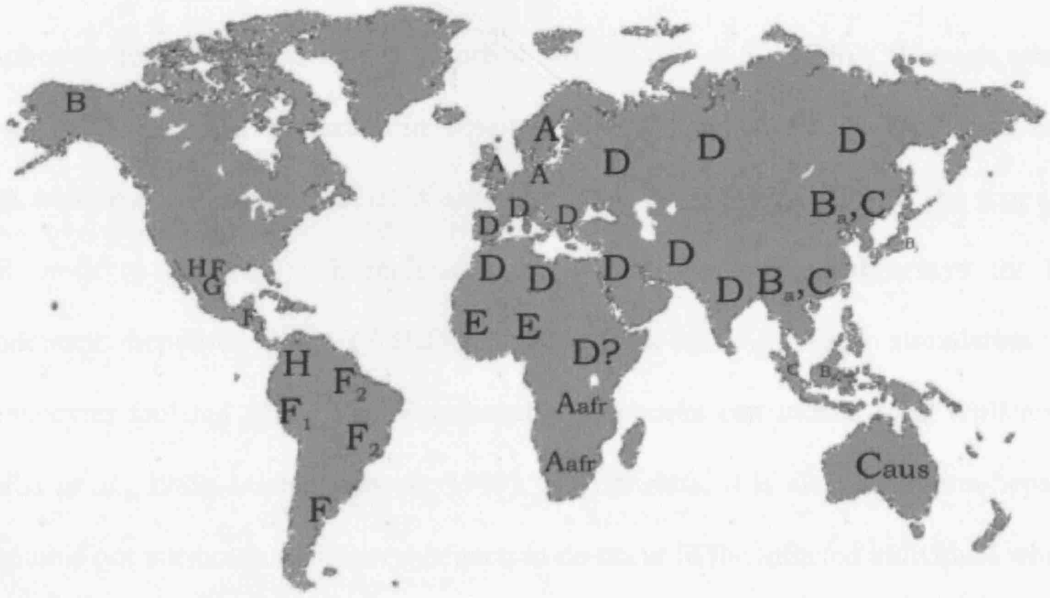


Figure 1.5 – The global distribution of HBV genotypes. (Schaefer, 2005)

1.7: Replication and life cycle of HBV

In the 35 years since Dane particles were observed, there has been huge progress in understanding of the later stages in the viral life cycle. However what goes on in the earlier stages of the cycle, namely, virus binding and internalisation, remains obscure. This lack of progress is attributable largely to the lack of a system in which the virus can be cultivated.

As hepatocytes are the major cell type in the liver, it would be anticipated that they are the major targets of infection by a hepatotropic virus such as HBV. Indeed, hepatocytes so far constitute the only cell type in which replication of all hepadnaviruses occurs (Seeger and Mason, 2000). Biliary ductal cells, epithelial cells, as well as subsets of cells in the pancreas, kidneys and lymphoid system may also be targets of infection (Seeger and Mason, 2000). The evidence for such extrahepatic sites of replication is, however, far from conclusive, with much work being conducted for duck hepatitis B virus (DHBV) rather than HBV. Some studies have suggested that HBV observed in lymphocyte preparations is due to absorbed virus (Kock *et al.*, 1996), although others show evidence of viral replication in these cells, i.e., the presence of cccDNA, and viral RNA transcription from the cccDNA template (Stoll-Becker *et al.*, 1997). The scope of PCR work to confirm such replication is limited, but biological assays for the woodchuck hepatitis virus (WHBV) have shown that mitogen stimulation of lymphocytes isolated from WHBV infected woodchucks can induce viral replication (Korba *et al.*, 1989; Michalak *et al.*, 1999). Nevertheless, it is clear that extra-hepatic symptoms not attributable to liver dysfunction do occur in the infected individual which may be due to extra-hepatic replication, although the role of antigen-antibody complexes cannot be excluded (Seeger and Mason, 2000).

The initial interaction between viruses and their host cell is an important determinant of virus tropism. HBV exhibits narrow host tropism and an even more narrow tissue specificity. The human HBV has been shown to be infectious only in humans, chimpanzees, gibbons (and possibly certain macaques) (De Meyer *et al.*, 1997). Even for this small number of host species, almost all viral replication is limited to hepatocytes – although limited replication may occur in other sites (Mason and Seeger, 2000) (as discussed earlier). The tropism may in part be determined by the cellular receptor with which HBV interacts. No receptor has yet been identified, however, which may be due to the fact that there are no cell lines available that are susceptible to HBV infection. Some cell lines (such as HepG2 and Huh7) can support HBV replication upon transfection of the viral genome but their usage is unsuitable for characterising the receptor.

Some progress has been made however in identifying the part of the viral surface which interacts with the cellular receptor. Accumulated evidence suggests that the region of the viral envelope that interacts with the cell is the preS1 domain (Neurath *et al.*, 1986; Pontisso *et al.*, 1989). Despite this evidence, it has not been possible to discount the possibility of interactions between the preS2 (De Meyer *et al.*, 1997) and the small S (Hertogs *et al.*, 1993) domains, though it is difficult to reconcile the concept of extremely high circulating levels of HBsAg with cellular receptor interactions, as this may have the consequence of blocking circulating virion attachment.

Penetration of virus into the cell is also not well understood. This step has not been easy to investigate with the lack of cell culture systems. PCR methodologies, designed specifically to amplify "across the gap" of the partially double stranded genome (Kock *et al.*, 1996) do exist, but their reliability remains in doubt. Whilst the selective

amplification of the replicative intermediate cccDNA is commonly used in penetration studies, such data can often be of limited value. Typically confirmation of the results produced in a replication study requires the purification of viral DNA replicative intermediates that are often present at only extremely low levels, making interpretation tenuous.

Better progress has been made in understanding the later steps of replication. Summers and Mason (1982) reported the landmark discovery that viral DNA replication proceeds not by conventional semi-conservative DNA synthesis but by reverse transcription of an RNA intermediate. The work was based on subviral particles prepared from DHBV-infected liver. These particles incorporated labelled deoxynucleosides (dNTPs) into both plus- and minus- strands of viral DNA, which would be anticipated from the action of a DNA polymerase. Critically, the synthesis of minus-strand DNA was resistant to actinomycin D, implying that its template was not DNA (plus-strand synthesis is sensitive to this compound). In addition, a portion of newly made minus-strand DNA was found in the form of RNA-DNA hybrids. These observations suggested that minus-strand DNA was made from an RNA template, which by inference, had been removed. Such an RNA was indeed identified soon thereafter (Buscher *et al.*, 1985; Enders *et al.*, 1985; Moroy *et al.*, 1985) and designated pregenomic RNA. It was also demonstrated that replication of HBV was asymmetric and that a reverse transcription replication, similar to that of DHBV, also applied to HBV (Blum *et al.*, 1984; Fowler *et al.*, 1984).

The replication scheme proposed by Summers and Mason (1982) has been largely confirmed. The HBV replication cycle may be summarised as follows. Upon entering the hepatocyte the viral envelope is stripped, and the core, containing the partially double stranded DNA, transported to the nucleus. At this stage the HBV DNA is in the

non-replicative, relaxed circular form. The short positive strand (S+) is completed by the HBV DNA polymerase and the completed DNA converted to the ccc form, which is suitable for template transcription. Transcription of the minus-strand produces several RNAs that may vary in length. A 3.4 kb RNA is produced which codes for the major structural core protein (p22), the reverse transcriptase protein and the polypeptide primer required for minus-strand synthesis. There are also a 2.4 kb and an approximately 2.1 kb RNA that have heterogeneous 5' ends. All of these RNAs have fixed 3' polyadenylated ends that are mapped from the start of the core gene. The 3.4 kb RNA is transcribed and serves as a template for HBV replication. A short sequence was identified at the 5' end of the RNA pregenome which is required for encaspidation (Junker-Niepmann *et al.*, 1990) and it was determined that a fully functional polymerase gene product is required for encapsidation (Bartenschlager *et al.*, 1990). Once encapsidated, the reverse transcriptase, primed by the virus encoded terminal protein at the DR1, transcribes a complementary (minus) strand of DNA. Meanwhile, the RNAaseH progressively degrades the RNA template from its 3' end leaving only a short 5' ribonucleotide. This ribonucleotide is then cleaved and translocated to the DR2 on the minus strand DNA where it serves as a primer for the DNA polymerase to transcribe a DNA plus-strand. Some of the core particles, containing newly synthesised viral DNA, are recycled back into the nucleus to amplify the pool of HBV genomes available for transcription. The remainder is assembled into virions before the plus-strand of the genome has been completed. The mature core particles are then packed into the HBsAg/preS endoplasmic reticulum and exported from the cell.

1.8: HBV proteins

1.8.1: Envelope proteins

The utilisation of three separate initiation codons (AUG) in the ORF S/preS1/preS2/S allows three carboxyl co-terminous proteins to be encoded. The envelope proteins therefore exist in three forms, a small (SHBs), medium (MHBs) and large (LHBs). The initiation site located furthest downstream encodes the smallest protein product, SHBs, comprised of 226 aa and referred to as P24, or when glycosylated (at codon Asn 146), as GP27. The second initiation codon is located 165 bases upstream of the first and is responsible for transcription of the MHBs protein (P33 and GP36), which consists of SHBs with a 55 aa extension provided by preS2. The third start codon encodes a further 108-109 aa extension to produce the LHBs protein (P39 or GP 42), which includes the preS1 extension. The LHBs therefore contains all three domains. – S, preS2 and preS1. All three envelope proteins are glycosylated type II transmembrane proteins. They form multimers stabilised by disulphide bridges formed between cysteine amino acids within the S domain (Seeger and Mason, 2000). The LHBs is further modified by N-terminal myristylation which has been shown to be essential for infectivity, but not for virus assembly (De Falco *et al.*, 2001).

The LHBs and MHBs proteins are present in roughly equal amounts in Dane particles and together constitute roughly 30% of the envelope content whilst the SHBs protein is present in around a 10-fold excess of the other two (Heermann *et al.*, 1987). SHBs can itself or in conjunction with larger envelope proteins form spherical and filamentous surface antigen particles, although filamentous particles generally contain about 20% LHBs whilst spherical particles are almost exclusively SHBs. The MHBs protein is found in about 10% of all forms (Tiollais *et al.*, 1985). HBsAg particles are secreted

from infected cells in at least 100-fold excess over virions and can reach concentrations of several hundred picograms per milliter in the blood of infected patients (Seeger and Mason, 2000).

The weight of evidence suggests that epitopes on preS1 may be involved in interaction with the host cell receptor (Neurath *et al.*, 1986; Pontisso *et al.*, 1989), although others in preS2 and the SHBs domains may also be involved (De Meyer *et al.*, 1997; Hertogs *et al.*, 1993). HBsAg epitopes displayed externally on the virion can alter the host range upon recombination (Ishikawa and Ganem, 1995). The preS1 domain also provides the ligand for core particles during assembly of the viral envelope. The function of the MHBs protein is less clearly understood, and the preS2 domain is apparently not essential for successful infection of hepatocyte cultures (Fernholz *et al.*, 1993).

Study of HBsAg soon after its discovery revealed a degree of serological heterogeneity (Le Bouvier, 1971). Four major antigenic determinants of HBsAg can be distinguished with antibodies that recognise different epitopes formed by SHBs. All the known subtypes contain the *a* determinant, which is encoded between amino acids 124-147 (Ashton-Rickardt and Murray, 1989). The mutually exclusive subtype specific determinates *d/y* and *w/r* can be inferred on the basis of amino acid changes from K to R at amino acids 122 and 160 respectively (Bancroft *et al.*, 1972; Peterson *et al.*, 1984; Okamato *et al.*, 1987). Additional subdeterminants have been found, allowing the differentiation of four serotypes of *ayw* and two of *adw*, but the additional *q+/q-* determinants on the *adr* subtype brings the total number of recognised serotypes to 9: $ayw_{1\text{-}4}$, *ayr*, adw_2, adw_4, *adr* q^- and *adr* q^+. These subtypes do not precisely correlate with genotypes (Okamato *et al* 1988; Norder *et al.* 1992; Norder *et al* 1993; Norder *et al* 2004; Magnius & Norder 1995).

The antigenic structure of HBsAg

The HBsAg protein contains B, T helper and CTL epitopes. Antibodies produced during infection and immunisation are directed against multiple epitopes of HBsAg. Both linear and discontinuous B-cell epitopes are known to exist on the HBsAg.

The precise spatial structure of HBsAg is not as yet well characterised. Computer modelling has identified three hydrophobic and two hydrophilic domains (Prange *et al.*, 1995; Prange and Streeke, 1995). It is thought that the first two hydrophobic domains play an important role in anchoring the S protein to the membrane (Bruss and Ganem, 1991). The third hydrophobic domain was predicted to lie between surface amino acids 161-226, and it was demonstrated that the HBs protein can tolerate a 51-aa deletion within this region, and still be secreted (Prange *et al.*, 1995). However, HBsAg that has been truncated at the C-terminal has been found to be secretion deficient (Bruss and Ganem, 1991).

The two predicted hydrophilic domains are found between aa 30-80 and 99-169. It is suspected that the first hydrophilic domain lies on the inner surface of the viral envelope and may be involved in attachment of the core particle during virion maturation (Prange *et al.*, 1995). The second hydrophilic loop is exposed on the virion and HBsAg particle surface and contains the major group and subtype-specific determinants. The region has a highly complex structure and is particularly cysteine rich. This provides the potential for secondary, tertiary and quaternary structures to develop through cysteine-cysteine disulphide bridges. There is still no consensus as to the precise structure of this region, and competing structural models have been proposed. It is, however, clear that the disulphide bonds are crucial in the conformation of this region. The 'double loop'

model proposes two loops, at surface amino acids 124-137 and 139-147, which are stabilised by disulphide bonds (figure 1.7). This model is supported by work with circularised synthetic peptides, which are though to mimic the loop structure (Brown *et al.*, 1984).

The studies of mutations within this region, both iatrogenically induced and those induced by site-directed mutagenesis, have allowed greater appreciation of the structure of this region (Ashton-Rickardt and Murray, 1989). The substitutions C124S, K141E, P142G, P142I and C147S have been found to lead to a reduction in binding to anti-HBs (Steward *et al.*, 1983). Such consequences would be expected, as cystine amino acids are known to be involved in disulphide amino acids, and proline is an important amino acid that determines protein conformation. The immune escape G145R mutant (Carman *et al.*, 1990) can cause considerable sterochemical change. Glycine amino acids are frequently located at junctions between domains or turns into polypeptides, and this particular amino acid appears to be critical to maintain the correct conformation for the postulated second loop.

Work with the phage display system has allowed the development of another model of this region (Chen *et al.*, 1996). This work confirmed the discontinuous nature of this epitope, as some monoclonal antibodies recognised amino acids that were 70 aa apart. This model proposed disulphide bridges between C121 to C124, C139 to C147, C107 to C138 and C137 to C139, although the latter two were considered less significant.. In contrast, another study demonstrated that the C149 amino acid to be important for antigenicity (Bruce *et al.*, 1995). This lack of consensus, particularly to the C-terminal of HBs, highlights that the fine structure of this region remains to be resolved. The model of Chen *et al.*, 1996 can be seen below in figure 1.6.

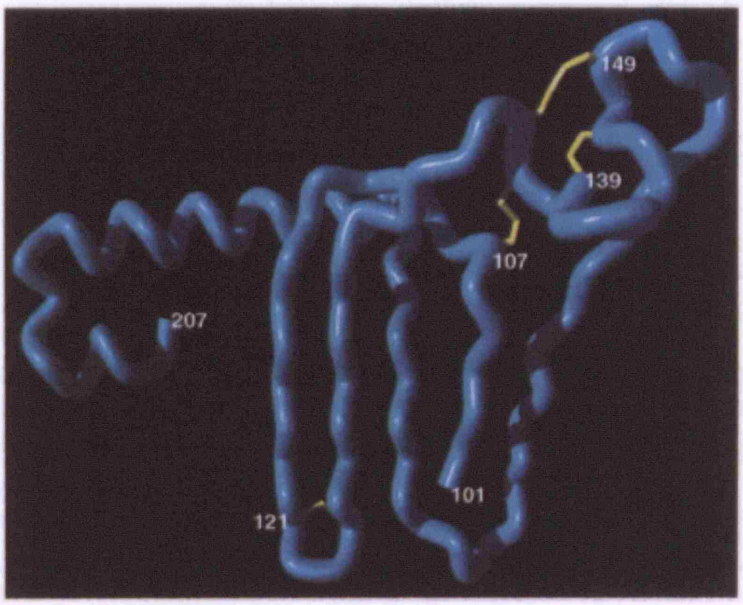


Figure 1.6 – A plausible 3 dimension structure for HBsAg. (Chen *et al.*, 1996).

An alternative approach to looking at antigenicity focuses less on the *a* determinant and considers the entire area covering aa 99-169, named the Major Hydrophilic Region (MHR) (Wallace and Carman, 1997)(figure 1.7). It was proposed that the MHR be divided into 5 regions, named HBs1-HBs5. HBs1 would include the region upstream of aa 120, HBs2 between aa 120-123, HBs3 between aa 124-137, HBs4 between aa 139 and 147, and HBs5 from aa 148-169. The rationale for considering the entire region is sound, as despite the fact that the "a" determinant is clearly the immunodominant region (Howard *et al.*, 1984; Zheng *et al.*, 2004) mutations outside "a" can abrogate anti-HBs binding; it was observed that mutations in amino acids 110-118 and 154-158 can lead to poor detection in commercially available Abbot Ausria II radioimmunoassay (Carman *et al.*, 1997b). Such data was confirmed by the finding that mutations at amino acids 116, 118, 120, 159, 184 and 184 can also lead to vaccine escape in previously

immunised infants (Chong-Jin *et al.*, 1999). Moreover, mutations at amino acids 118 and 120 led to a reduction in mutant HBsAg binding to a panel of 6 MAbs (specific to the "a" determinant) in in-house solid phase capture sandwich ELISAs (Kfoury-Baz *et al.*, 2001) and mutations at S gene residues 105, 110, 112, 118 119 and 120 have been associated with reduced antibody binding (Jeantet *et al.*, 2004; Wagner *et al.*, 2004). It has also been seen that vaccine escape mutations occurring in the S gene, selected through therapeutic HBIG pressure in liver transplant patients, could also be seen to arise throughout the MHR, outside the "a" determinant in HBV S gene sequences isolated from patients who had failed HBV therapy (Terrault *et al.*, 1998). In this study HBV sequences from 4 patients who received high dose HBIG therapy but had subsequently failed to clear HBsAg were contrasted to patients who had cleared HBsAg and a non-HBIG receiving placebo group. Excluding the "a" determinant, it was shown that there were statistically more substitution mutations in the entire surface gene for the HBIG fail group than for the two control groups. The mutations were observed between surface residues 98-118 and 155-214 as well as the "a" determinant. This data infers that mutations in the S gene in regions other than that coding for the "a" determinant may be important in immune evasion. Furthermore, disruption of "a" determinant epitopes could also be observed with mutations that are more closely adjacent; thus, it was proposed that *d/y* specificity (determined by position 122) could be lost due to a substitution of the proline at amino acid 120 (Wallace *et al.*, 1994). Moreover, case reports have been presented which demonstrated that unusually, patients with chronic liver disease harboured mutations at surface codon 122 (which determines *d/y* specificity) in addition to other mutations of the *a* determinant and mutations out with the "a" determinant (Alexopoulou *et al.*, 2004; Alexopoulou *et al.*, 2006). In one instance the variant was undetectable by the Murex IMx and Murex AXSYM-MEIA commercial HBsAg assays (Alexopoulou *et al.*, 2006).

Another study found that a novel monoclonal antibody appears to bind amino acids 178-186 despite the fact that all models predict this region as buried within the lipid bilayer (Paulij *et al.*, 1999).

The findings of Paulij *et al.*, 1999 cast doubt on current HBsAg modelling data in the carboxyl terminus of HBsAg and serves as a reminder that there is still a lack of consensus about the antigenic structure of HBsAg, particularly downstream of the "a" determinant. The findings of Carman *et al.*, 1997b; Chong-Jin *et al.*, 1999; Kfoury-Baz *et al.*, 2001; Terrault *et al.*, 1998; Alexopoulou *et al.*, 2004; Alexopoulou *et al.*, 2006; Jeantet *et al.*, 2004 and Wagner *et al.*, 2004 all serve to highlight that there is still much to be understood about the precise interactions that occur between HBsAg epitopes and anti-HBs, particularly upon mutation. What is clear is that either some significant epitopes in the MHR lie outside (downstream) of the "a" determinant, or that amino acids outwith the "a" determinant can influence its conformation.

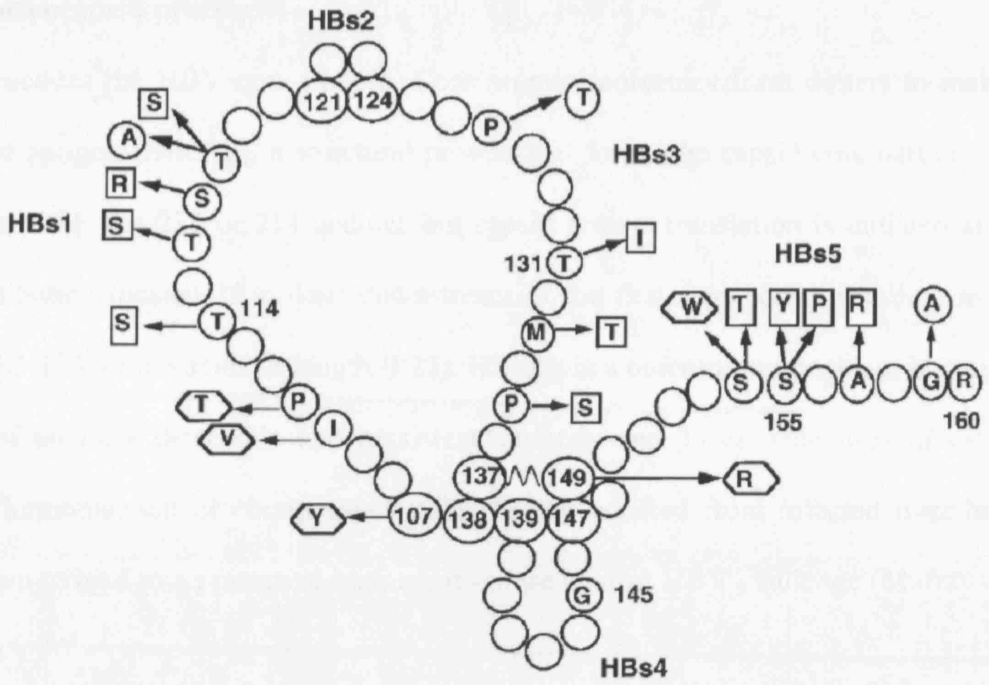


Figure 1.7 – Possible structure of the MHR, the "a" determinant showing typical mutations (Carman *et al.*, 1997b). This structure is derived from that generated by Prange *et al.*, 1995 and highlights the 5 MHR regions HBs1-HBs5 as proposed by Wallace and Carman, 1997.

1.8.2: Nucleocapsid proteins

ORF C encodes the HBV core protein. Core protein molecules form dimers to make HBV core antigen (HBcAg), a structural protein that forms the capsid core/particle of HBV. The ORF has 212 or 214 codons, but capsid protein translation is initiated at a second initiation located 29 codons downstream of the first start codon resulting in a protein 183-185 amino acids in length (P22). HBcAg is a potent immunogen in humans capable of eliciting detectable and persistent antibody and T-cell responses in most patients. Immunisation of chimpanzees with HBcAg purified from infected liver has been shown to lead to a protective immune response against HBV challenge (Murray *et al.*, 1987).

P22 is produced and phosphorylated in the cytosol of infected cells (Roossinck and Siddiqui, 1987) and polymerises to form HBcAg. It is required absolutely for packaging its own mRNA and for the viral polymerase after the formation of the mRNA complex. Thereafter, it assembles into core particles that also contain a cellular protein kinase C. Following DNA synthesis, the assembled core particles acquire envelopes containing the 3 envelope proteins from the endoplasmic reticulum. HBcAg is also thought to play a regulatory role in aspects of HBV replication (Nassal, 1992; Guidotti *et al.*, 1994).

The upstream region of ORF C is referred to as preC and in conjunction with ORF C it is responsible for the transcription of the second protein product of ORF C, the HBV e antigen (HBeAg), a secretory form of P22. HBeAg contains an addition 29 amino acids, which are predominantly hydrophobic. The preC sequence encodes a terminal α-helix, which acts as a secretory signal (Ou *et al.*, 1986; Standring *et al.*, 1988) allowing HBeAg to move into the lumen of the endoplasmic reticulum. During this translocation, the 19 amino terminal amino acids are removed by a signal peptidase. Further cleavage

of 34 amino acids occurs at the carboxyl terminal, which results in the HBeAg molecule of 159 amino acids (Garcia *et al.*, 1988). Thereafter HBeAg is transported to the Golgi apparatus and then secreted from the cell or transported to the nucleus (Ou *et al.*, 1986; Ou *et al.*, 1989; Uy *et al.*, 1986; Wang *et al.*, 1991).

HBeAg is not required for viral infection or replication (Schlitt *et al.*, 1987). Indeed, variants deficient in HBeAg production arise naturally during infection. Such variants have also been noted to arise during interferon treatment of HBV infected individuals. HBe proteins are incapable of assembling into capsid particles despite the almost total homology of the cleaved protein with HBc protein due to the remaining 10 amino acids of the preC portion preventing HBcAg assembly by interacting with the preC sequence (Wasenaur *et al.*, 1992).

In acute infections, the appearance of HBeAg in patients occurs at approximately the same time as HBsAg. HBeAg is associated with continued virus replication and high infectivity. As HBcAg and HBeAg are cross-reactive to T cells as they have the same oligo-peptide sequences. The production of HBeAg may partially block the cellular immune responses against HBcAg and promote viral persistence. Circulatory HBeAg persistence is strongly associated with the development of HCC. Thus, HBeAg-seropositive Taiwanese patients chronically infected by HBV are ~60 times at risk of developing HCC compared to those who are seronegative (Yang *et al.*, 2002).

Usually after 10-20 years, HBeAg will be cleared from the circulation, an event associated with flare up in immune-mediated hepatitis, elevated liver transaminase levels and hepatocellular necrosis. This event is followed by the appearance of anti-HBe antibodies and a concomitant reduction of replication and circulating HBV DNA levels,

a state referred to as the "inactive carrier state". HBeAg seroconversion generally changes the natural history of chronic hepatitis B resulting in little or no ongoing liver damage. The association of HBeAg clearance with the partial clearance of HBV suggests that HBeAg may be an important immune target.

It was previously observed that the loss of HBeAg from the circulation may also result from the development of a dominant HBV variant that is incapable of producing HBeAg [a state often due to the presence of a mutation in the pre-core region at nucleotide 1894 or 1896] (Carman *et al.*, 1989). Such patients begin again to replicate HBV at high levels and will show raised transaminase levels after apparent (but false) HBeAg "seroconversion" during which HBV DNA levels dropped. The prognosis for patients infected with such "pre-core mutants" is poorer than for wild type HBV. It is also possible to find such pre-core mutations in the serum of asymptomatic HBV carriers (Kann and Gerlich, 2000). It is now appreciated, that rather than being a distinct, atypical disease state associated with sporadic pre-core mutations (as was previously thought), that in fact HBeAg negative CHB with pre-core mutations is an inevitable late stage reactivation of CHB infection that develops after HBeAg loss and appearance of anti-e (Hadziyannis, 1995; Yim and Lok, 2006).

1.8.3: X protein

The HBx protein encoded by ORF X is apparently not essential for any stage in the viral life cycle in transfected cells (Blum *et al.*, 1992) but is required for the establishment of infection *in vivo* (Zoulim *et al.*, 1994). The X gene is thought to be an early gene (Wu *et al.*, 1991). The gene is conserved in similar form in the hepadnaviruses of woodchucks and ground squirrels (although there is no such homologue in the avihepadnaviruses,

implying that the HBx protein is not required for replication or particle assembly in other hepadnaviruses). The protein of 17kDa is encoded by a gene spanning 154 aas and can be detected in the serum from patients with chronic HBV infection, in whom there may be a correlation with severity (Feitelson & Clayton 1990). However much is still unknown about the precise function of the protein, though its main function is to act as a transcriptional activator (it has been known to transactivate a number of cellular and viral promoters [Rossner, 1992]), and it has been postulated to play a role in interacting with the p53 tumour suppressor protein, deregulation of cell cycle check points, and the abrogation of p53-dependant apoptosis (Arbuthnot *et al.*, 2000).

Several mechanisms by which HBx could perform its transactivating function have been proposed. HBx does not bind directly to DNA and hence requires the association with cellular proteins NFkB, TFIIIC and AP2 (Maguire *et al.*, 1991; Unger *et al.*, 1990; Seto *et al.*, 1990). Similarities with a Kunitz type serine protease inhibitor lead to the speculation that HBx is also a protease inhibitor that may play a role in carcinogenesis (Takada *et al.*, 1990). Evidence for a serine/theorine protein kinase activity of HBx has been reported, thereby implicating its role in hepatocellular-carcinogenesis (Wu *et al.*, 1990). HBx was shown to induce liver cancer in one line of transgenic mice (Kim *et al.*, 1991), though work performed in another line observed no tumourogenesis (Lee *et al.*, 1990).

Most patients infected by HBV develop antibodies to HBx. These antibodies are produced very early in infection, but may fall to undetectable levels in self-limited hepatitis, and reach the highest titres in chronic carriers with ongoing viral replication (Levrero *et al.*, 1990).

1.8.4: Polymerase protein

The longest ORF is the P ORF, which consists of 4 distinguishable domains (Schlicht *et al.*, 1991) and is a large protein of up to 845 aas. The first (amino terminal) domain encodes a primase, which remains covalently linked to the 5' end of the minus strand of the viral genome; this enzyme is responsible for the priming of minus strand synthesis (Wang and Seeger, 1992; Weber *et al.*, 1994; Zoulim and Seeger, 1994). The second domain functions as a spacer between the first and third domain and appears to have no other function. The third domain encodes the viral polymerase/reverse transcriptase, a protein of approximately 90kDa. Sequence analysis of the reverse transcriptase domain has revealed homology with the corresponding *pol* gene of retroviruses (Toh *et al.*, 1983). The polymerase protein is packaged together with the viral pre-genome into core particles. The polymerase activity is RNA- or DNA-dependant. The carboxyl domain encodes an RNAaseH, which is responsible for cleaving the RNA, is associated with the genome as an RNA: DNA hybrid (Schlicht *et al.*, 1991).

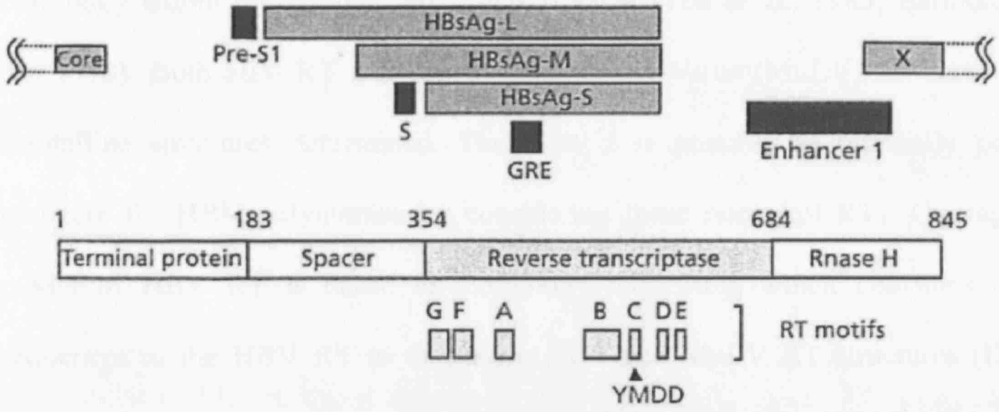


Figure 1.8 - Reverse transcriptase domain structure. (Delaney *et al.*, 2001). Numbers indicate polymerase amino acid number. YMDD is the conserved amino acid motif of the reverse transcriptase domain. A-G indicate conserved motifs that are essential functional domains.

The reverse transcriptase domain of HBV polymerase contains seven conserved motifs (A-G) that have extensive homology with other DNA polymerases (figure 1.8). These regions are sometimes termed "subdomains", this terminology does not refer to the fingers, palm and thumb domains which are standard features in HBV polymerase as with all in all polymerases (Delaney *et al.*, 2001). In HBV polymerase subdomains G, F, A and B belong to the fingers domain, C, D and E to the palm domain (figures 1.8 and 1.9) In order to describe conveniently the location of mutations within this region, and to cope with inter-genotypic variability, the conserved EDWGPCDEHG domain at the N-terminus of the reverse transcriptase (RT) domain is taken as the beginning of the reverse transcriptase domain numbering system; such a measure has allowed the

standardisation of aa changes (Stuyver *et al.*, 2001). HBV polymerase has not been crystallised and so the precise structure of the RT domain remains unclear. There is homology within retroviral reverse transcriptases (Toh *et al.*, 1983; Bartholemuesz *et al.*, 1998). Both HIV RT and Murine Leukaemia Virus (MuLV) RT have had their crystalline structures determined. Therefore it is possible to rationally postulate a structure for HBV polymerase by considering these retroviral RTs. One appropriate model of HBV RT is based on homology modelling, which considers conserved sequences in the HBV RT to the known HIV and MuLV RT structures (Das *et al.*, 2001) (figure 1.9), and has been used to predict the nature of some mutations that specify resistance of HBV to antivirals (Yang *et al.*, 2004).

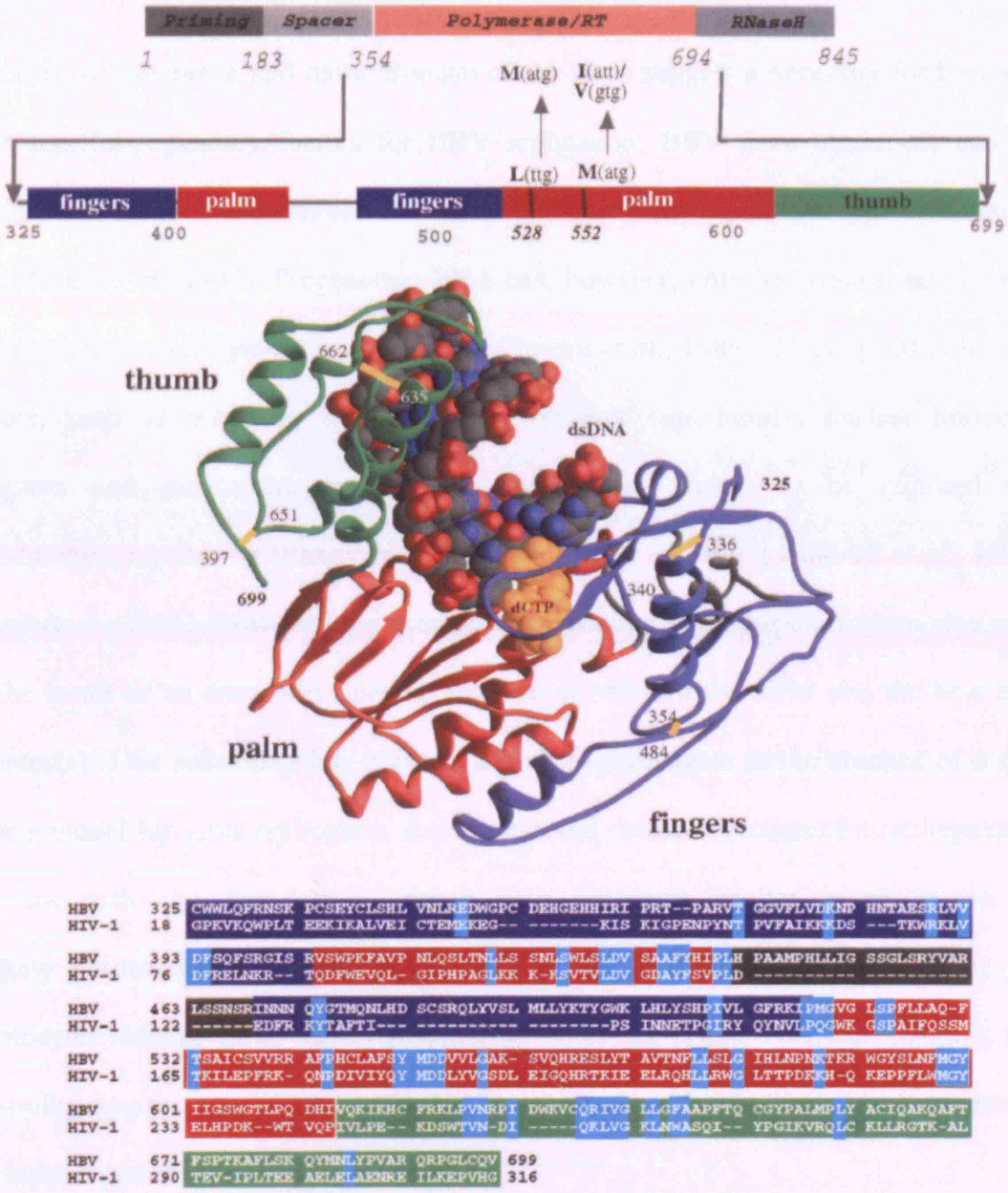


Figure 1.9 – Homology model of HBV reverse transcriptase (Das *et al.*, 2001). The reverse transcriptase numbering system used is that which preceded the standardised system proposed by Stuyver *et al.*, 2001.

1.9: Significance of the lack of a cell culture system for HBV

The narrow host range and tissue tropism of the virus suggest a necessity for host and liver specific regulatory factors for HBV replication. HBV gene transcripts can be produced in a wide variety of cell lines derived from various species and diverse tissues (De Meyer *et al.*, 1997). Pregenomic RNA can, however, only be synthesised in well-differentiated hepatocyte-derived cell lines (Sureau *et al.*, 1986). Liver specific nuclear factors, such as members of the nuclear receptor superfamily, nuclear hormone receptors and peroxisome proliferators, have been shown to be required for hepadnavirus replication (Raney *et al.*, 1995; Raney *et al.*, 1997; Guidotti *et al.*, 1999; Raney *et al.*, 2001). However, the extreme host specificity and hepatotropism also may be the result of an extremely specific interaction between the virus and the host cell receptor(s). This possibility has proved difficult to investigate in the absence of a cell culture model for virus replication. It was observed that transfection of a rat hepatoma cell line with a greater-than-full-length HBV construct resulted in production of progeny virions, although both *in vivo* and *in vitro* infection experiments using rat hepatocytes resulted in no viral replication (Shih *et al.*, 1989). This data suggests the possibility that the species barrier for HBV replication and infection which operates at the early stages of infection.

The practical difficulties arising from the inability to culture virus are scientifically and clinically significant. Although there has been huge progress towards understanding of the basic biology of the virus and in the treatment of infections, that progress can in some circumstances effectively be limited by the absence of a cell culture system supporting virus replication as researchers are forced to perform circuitous experiments

to retrieve data which for other viruses would be easily accrued through cell/virus culture.

1.9.1: Animal models and the limits of the DHBV model

Many of the basic virological investigations have taken place in animal models, often utilising the avian hepadnaviruses, which differ fundamentally, at a molecular and a biological level, from the mammalian hepadnaviruses. Avihepadnaviruses encode only two envelope proteins – there is no equivalent of the preS2 domain. There is also no homologue of the mammalian HBV X gene in avihepadnaviruses. Furthermore, avian hepadnaviruses have been shown to have distinct transcriptional requirements which differ from mammalian hepadnaviruses (Tang and McLachlan, 2002), whilst DHBV or HBV replication can be supported in non-hepatoma cells, woodchuck hepatitis virus (WHBV) replication cannot (Tang and McLachlan, 2002). This makes some aspects of extrapolation from avian HBV to mammalian viruses, and within mammalian viruses, difficult. More recently, it has been observed that the Tupaia (tree shrew) is susceptible to HBV infection and can undergo an acute HBV associated illness. This animal thus may provide a much needed more manageable small animal model for HBV infection studies (Walter *et al.*, 1996).

1.9.2: Primary human hepatocytes and HBV studies

It is possible to make use of primary human hepatocytes, which are, for a short time, susceptible to HBV (Seeger and Mason, 2000). Primary hepatocytes are nevertheless difficult to obtain and of variable quality. Furthermore, the hepatocytes lose susceptibility to the virus within a relatively short period of time. In addition, in many

cases where the infection of hepatocytes has been reported, the hepatocytes have been maintained in medium containing chemical agents, such as polyethylene glycol or dimethyl sulphoxide, which may modulate the mechanism of entry of the virus into cells, possibly utilising "unnatural" mechanisms of entry (Gripon *et al.*, 1988; Pugh and Summers, 1989; Gripon *et al.*, 1993).

1.9.3: Transfection of greater-than-full-length constructs and single genes

Many virologists have resorted to using an artificial system whereby the binding and internalisation of the virus into the host cell is bypassed. Studies of this type utilise the ability of hepatocyte derived cell lines, such as HepG2 and Huh-7 cells, to support the replication of the virus. Once viral DNA reaches the nucleus, these cell lines are able to provide the necessary "cellular machinery" for HBV gene expression DNA replication, packaging and production of progeny virions. Virion cell entry problems can be avoided through the artificial transfection of greater than full-length genomes from which virus particles may be produced (Acs *et al.*, 1987). Constructs containing the-greater-than-full-length genome are required for production of a full-length terminally redundant pregenome, which would normally be transcribed from the circular genome. Although this system has been used successfully to elucidate much of the biology of HBV, there are also obvious limitations; such approaches are unable to deliver data about the entry stage of infection. The development of a more "natural" cell culture system would therefore be extremely valuable in investigating the very early events in the virus replicative cycle.

The same limitations apply to the use of cell lines that constitutively express the HBV genome. The two most commonly used cell lines are the HepG2.215 cell line and the PLC/PRF/5 cell line, sometimes referred to as the "Alexander" cell line. The

PLC/PRF/5 cell line is a human hepatoma cell line that constitutively secretes HBsAg (McNab *et al.*, 1976) and can proliferate in the presence of antibody against HBsAg (Alexander *et al.*, 1978). The cell line has been shown to contain 4 complete and 2 partially integrated HBV genome copies (Marion *et al.*, 1980; Edman *et al.*, 1980). Despite the presence of surface mRNA transcripts, no core mRNA transcripts have been detectable. Infectivity experiments with chimpanzees have been undertaken to demonstrate that the cell line does not secrete infections virus (Daemer *et al.*, 1980). The cell line is extremely valuable allowing some experiments on the production and the expression of HBsAg to be undertaken. However, the titres of HBsAg secreted by cells are generally low, so the cell line is not a reliable source of large quantities of HBsAg.

The HepG2.2.15 cell line is a human hepatoblastoma derived cell line which has been deliberately transfected with multiple copies of the HBV genome, which is both integrated into the host cell genome and maintained as an episome (Sells *et al.*, 1987). Unlike the Alexander cell line, the HepG2.2.15 line constitutively secretes HBV particles and so the study of this cell line has been particularly useful in studying the later stages of HBV replication and in the screening of antivirals which may inhibit HBV replication (Korban and Gerin, 1992).

More recently, a hepatocellular carcinoma-derived cell line, FLC4A10II, has been developed; it is a stable transfected cell line containing multiple copies of the viral genome, with no observable ill effects on the host cells, and is able to secrete HBsAg, HBeAg, HBV DNA and infectious virions (Fellig *et al.*, 2004).

However, as both the FLC4A10II and HepG2.2.15 cell lines contain only wild type HBV, they are not useful for analysis of resistant mutants; in such instances the use of transfection techniques is more appropriate.

The seminal discovery of the method to produce recombinant DNA (Cohen *et al.*, 1973) paved the way for researches to construct biologically active plasmids and other DNA constructs containing cloned genes of interest. This has allowed the discipline of reverse genetics to flourish. Single viral genes can easily be cloned into prokaryotic, eukaryotic and viral expression vectors. Cells can be transfected with vectors containing cloned genes and so proteins can be produced on demand for study. However, care must be taken by the researcher to ensure that the novel recombinant protein is sufficiently analogous to the real wild type protein. For example, recombinant viral envelope proteins produced in bacteria will not be glycosylated and so may have altered antigenicicty and/or conformation. Recombinant DNA methods have allowed eukaryotic cells to synthesize HBsAg, which is assembled into and secreted as a complex structure (as a 22- nm particle) and is indistinguishable from that formed naturally during human infection (Liu *et al.*, 1982; Hitzeman *et al.*, 1983). This approach, in conjunction with site directed mutagenesis, also has great potential to facilitate the study of genetic variants of each HBV ORF.

1.10: Serological profiles of HBV markers

Several of the HBV serological markers provide a basis for monitoring the appearance of the virus and immune response during the course of infection. The profiles of these serological markers can be correlated with the course of disease and hence offer useful diagnostic and prognostic information.

1.10.1: Serological markers in acute HBV infections

The typical incubation period in a naturally occurring infection is 3 months, although in post-transfusion hepatitis B it may be shorter. The first serological marker to appear is

HBsAg. This is typically detected from around 2 weeks post infection prior to the acute stage of illness, before declining to undetectable levels within 4 to 6 months. HBV DNA levels rise early in the incubation period (coinciding with the first appearance of HBsAg) and is known to correlate with serum HBV polymerase activity (Kaplan *et al.*, 1974). At this early stage HBeAg and HBV DNA can be detected also. Upon the onset of clinically apparent hepatitis, levels of DNA polymerase and HBeAg rapidly decline (Aldershvile *et al.*, 1980). Typically, antibody to HBcAg (anti HBc) appears in the sera of infected individuals soon after the detection of HBsAg. The IgM isotype of anti-HBc is the first antibody to appear in serum (Cohen, 1978) and can persist for 6-24 months (Hatzakis *et al.*, 2006). It has been noted that anti-HBc IgM levels were higher in patients with an acute resolving infection than in patients who had developed chronic infection (Tedder and Wilson-Croome., 1981). As the illness progresses and HBsAg clears, anti HBcAg IgM is replaced by IgG. Anti-HBc can persist for many years and is a diagnostically useful marker of HBV infection between the disappearance of HBsAg and the development of anti-HBs. Anti-HBs is a serological marker that is ultimately demonstratable in more than 90% of patients who experience a primary infection; once present it usually persists for many years and is protective against further attacks of wild type hepatitis B (Krugman *et al.*, 1979). The infectivity of a patient is related to the presence or absence of circulatory HBeAg and anti-HBe. The presence of HBeAg is generally regarded as being consistent with a high level of infectivity and correlates well with the presence of serum detectable HBV DNA (Krugman *et al.*, 1974; Krugman *et al.*, 1979; Hoofnagle *et al.*, 1981). As discussed earlier (section 1.8.2) relatively high viraemia can still be found in some patients infected by pre-core mutants. HBeAg is rapidly lost during recovery from an acute infection and anti-HBe develops early in the convalescent phase. The presence of serum anti-HBe is generally taken as an indication

of low infectivity particularly in carriers. The duration of this antibody response is shorter than that of anti-HBc and anti-HBs.

1.10.2: Serological markers in persistent HBV infection

While the time course of the typical (acute) HBV infection is measured in months, in some patients the natural history of the infection evolves over many years. These chronically infected patients are termed HBV (or HBsAg) carriers and are identified by the persistence of HBsAg at stable levels for >6 months.

There are four distinct phases of chronic HBV infection (figure 1.10) these are; (i) the immune tolerance phase, (ii) the immune clearance phase, (iii) the inactive carrier state and (iv) reactivation.

(i). Patients who acquire HBV through the perinatal route typically experience a long immune tolerance phase which may last between 1-4 decades. It is characterised by the presence of HBeAg, high levels of serum HBV DNA, normal serum ALTs and minimal or no inflammation on biopsy. Only a small percentage of patients experience cirrhosis and almost none develop HCC (Yim and Lok, 2006). Patients who acquire HBV later in life experience only a very short or entirely absent immune tolerant phase and in these patients the initial development of the carrier state parallels that of acute infection.

Levels of HBsAg, HBeAg and anti-HBc rise, but rather than HBsAg and HBeAg then declining like in acute infection, they may persist for many years. The anti-HBc response is much increased (Hoofnagle et al., 1975) with the production of high concentrations of IgG and small amounts of IgM,

(ii). The second phase is characterised by high levels of HBeAg, high or fluctuating serum HBV DNA levels, intermittent elevation in serum ALTs and active inflammation

on liver biopsy. The hallmark flares in ALTs are believed to be due to immune mediated lysis of hepatocytes as a consequence of increased T-cell responses to HBcAg (Bertoletti and Gehring, 2006). When the patient undergoes a hepatic flare IgM titres may rise (Liaw *et al* 1985, Mels *et al* 1994; Bortollotti *et al* 1990; Perillo *et al* 2001). The duration and severity of hepatic flares during the immune clearance phase correlates with the risk of cirrhosis and HCC (Yim and Lok, 2006). The variability in clinical presentations in infected individuals confirms that HBV does not play a direct cytopathic role (Dudley *et al.*, 1972) while immunological techniques have demonstrated that HBV related liver disease is immune-mediated, patients with chronic HBV infection have impaired immune response to HBV infection (Stoop *et al.*, 2005).

(iii). In the third phase, the inactive carrier state, HBeAg is subsequently lost and anti-HBe develops and the patient enters the inactive carrier state. This happens in approximately 10% of carriers per year (Weller *et al.*, 1986). Serum ALT levels are persistently normal and there is low or undetectable serum HBV. Liver biopsy may show mild hepatitis and minimal fibrosis, and some patients who accrued severe liver damage in the immune clearance phase may show inactive cirrhosis.

After a further period HBsAg may become undetectable with subsequent development of anti-HBs. However, some patients do not develop anti-HBs after the disappearance of HBsAg, though HBcAg, anti-HBc and HBeAg may still be markers of their infection.

The inactive carrier state may persist indefinitely, and in this state prognosis is generally favourable. However, some inactive carriers may undergo reactivation, either spontaneously, or as a result of immunosupression (Yim and Lok, 2006).

(iv). The fourth defined phase is the reactivation of HBV replication / HBeAg negative CHB and is characterised by negative HBeAg, positive anti-HBe, detectable serum

HBV DNA, elevated & fluctuating ALTs and continued necro-inflamation. Most patients reach this phase after a variable duration of the inactive carrier state, however some progress directly from HBeAg positive to HBeAg negative CHB. The development to HBeAg negative reactivation can be associated with the development of a dominant HBV variant that is incapable of producing HBeAg which may be present from phase 2 [a state often due to the presence of a mutation in the pre-core region at nucleotide 1894 or 1896] (Carman *et al.*, 1989). HBeAg negative hepatitis was originally recognised in Mediterranean countries, though now has been observed world-wide.

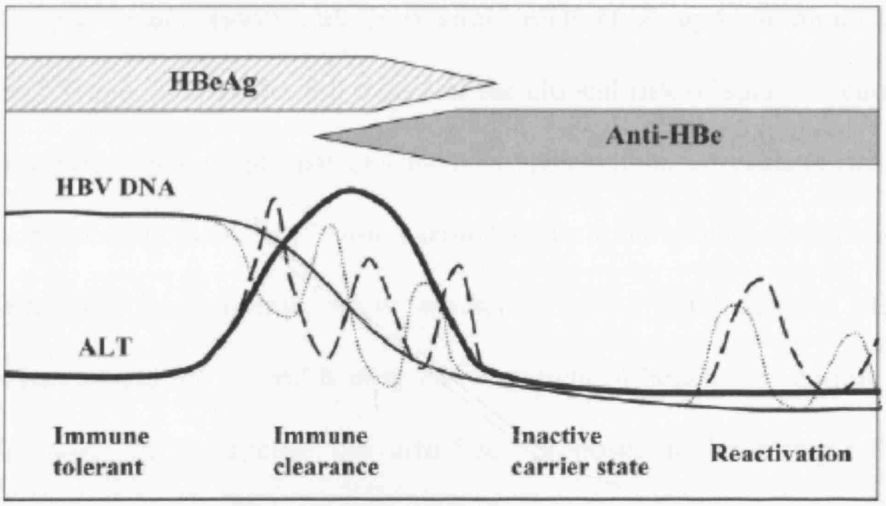


Figure 1.10 – The four phases of chronic HBV infection (Yim and Lok, 2006).

1.10.3: Serologically occult HBV infection

Some patients may develop a serologically occult hepatitis B infection, which is characterized by the detection of HBV DNA with undetectable HBsAg-seropositivity. In such instances, serum HBV DNA levels are typically very low, though antibody markers of viral infection such as anti-HBc and anti-HBe may still be present. The true cause of occult HBV infection remains unclear, but several possibilities have been hypothesized. These include: (i) mutations of HBV-DNA S gene sequence (Weinberger *et al.*, 2000); (ii) integration of HBV-DNA into host chromosomes (Lai *et al.*, 1990); (iii) infection of peripheral blood mononuclear cells by HBV (Bouffard *et al.*, 1990); (iv) formation of HBV-containing immune complex (Hu, 2002).; (v) altered host immune response (Hodgson and Michalak, 2001); (vi) interference of HBV by other viruses (Fukuda *et al.*, 1999) and; (vii) viral reactivation upon immunosuppression (Chemin and Trepo, 2005). The full extent of the clinical risk of such infection remains unclear; however it is thought that this form of HBV infection presents risks of HBV transmission through blood transfusion, haemodialysis and organ transplantation. It may cause cryptogenic liver disease, acute exacerbations of HBV disease, affect HBV disease treatment and the natural history and treatment of hepatitis C co-infection (Hu, 2002). Moreover, its emergence has also been proposed to be associated with the development of hepatocellular carcinoma (Paterlini *et al.*, 1990). Therefore the development of an occult infection in a previously typically chronically infected patient may result in a misdiagnosis; an infection may be misreported as cleared, when in fact it is not (Hu, 2002).

It is also necessary to consider non-virological possibilities for occult HBV infection. HBsAg assays may be lacking in sufficient sensitivity, or false positive HBV DNA results (potentially through contamination) may lead to the erroneous diagnosis of occult HBV.

1.11: Prevention of HBV infection

1.11.1: Active immunisation

It was observed that the anti-HBs response, which developed HBV infected individuals, prevented re-infection (Krugman, 1971). This phenomenon led to the realisation that the development of a strong, sustained anti-HBs response may be the key to the development of a successful vaccine. Initial vaccine preparatory efforts worked with crude immunogenic plasma preparations containing HBV and it was observed that these could prevent infection (Smuzness *et al.*, 1974). This work paved the way to the development of more sophisticated approaches for vaccination such as the use of highly purified, formalin and/or heat inactivated hepatitis B subvirion (22-nm) HBsAg particles free of detectable nucleic acid (Buynak *et al.*, 1976, Purcell *et al.*, 1975). The antigen was harvested from the plasma of asymptomatic, apparently healthy human carriers of HBV. Efficacy of the vaccine was demonstrated in a study undertaken amongst the staff and patients of a renal dialysis unit (Maupas *et al.*, 1978). The first commercially available vaccine was produced by Merck, Sharp & Dohme in 1982. The vaccine prevented HBV infection in all six chimpanzees that were challenged with HBV subtype *adw* (Hillman, 1979). Plasma-derived inactivated vaccine was subsequently found to be efficacious in a randomised, double blind, placebo controlled trial in a high-risk population of 1083 homosexual men (Szmuness *et al.*, 1980).

The emergence of the AIDS epidemic, in the course of which human immunodeficiency virus (HIV) transmission via blood products became a major problem, raised concerns regarding the safety of plasma-derived vaccines. To that end other HBV vaccine preparatory processes were investigated using recombinant DNA technology. As a consequence in 1986 the first yeast-derived vaccines were licensed. This recombinant

vaccine contained the S gene cloned into *E. coli* and expressed in yeast cells (Valenzuela *et al* 1982). The cross protective efficacy of vaccination using in vitro rather than in vivo derived HBsAg was demonstrated in chimpanzees (McAleer *et al.*, 1984), although this study itself used HBsAg-derived from a human hepatoma cell line that expressed HBsAg. Cross-protection by different serotype vaccines against various HBV subtypes was observed. The recombinant vaccine is still the basis of current licensed vaccine formulations such as Engerix B (GlaxoSmithKline), Recombivax HB (Merk), Genhevac B (Aventis Pasteur) and Hepavax Gene (Berna Biotech).

Novel vaccines have been developed which contain the preS1 and preS2 proteins. The proteins are incorporated as it has been observed that antibodies to both regions can be protective. Licensed examples include Hepacare (formerly Hepagene) [Powderject] and Bio-Hep B (Biotech.Gen.Corp). In Phase III development is a recombinant extra strength vaccine for poor responders, Extra Strength Hep B (GlaxoSmithKline/ Corixa). A DNA vaccine against HBV is currently in phase I clinical development - Hep B DNA vaccine Px (Powderject), though to date no results have been published.

Implementation of universal immunisation at birth has been shown to be effective in reducing the burden of disease attributable to HBV. Thus, in Taiwan a reduction in the incidence of hepatocelluar carcinoma is being observed (Chang *et al.*, 1997, Chang *et al* 2004). The WHO has recommended universal immunisation practices to all countries. Currently, 156 countries implement universal immunisation and of those 90 implement universal vaccination of newborns against HBV (Lai *et al.*, 2003). The current vaccine formulations are considered to be safe with no evidence of any long-term side effects (Lai *et al.*, 2003).

1.11.2: Passive immunisation

The administration of hepatitis B immunoglobulin (HBIG) pre- or post- exposure is effective in the prevention of HBV infection. Early studies with normal human immune globulin were inconclusive due to the lack of consistent levels of anti-HBs in preparations and by the possibility of the inclusion of HBsAg that would produce active-passive immunisation (Seeff *et al.*, 1978). HBIG, prepared from plasma that had been pre-selected for a high titre of anti-HBs (Szmuness *et al.*, 1974), effected protection solely by passive immunisation and was licensed for use in 1977.

With the availability of a vaccine for hepatitis B, HBIG is seldom used for pre-exposure prophylaxis. Situations in which post-exposure prophylaxis remains essential include (a) exposure of a susceptible individual to HBV- containing material by percutaneous inoculation or direct mucous membrane contact; (b) susceptible contacts of acutely infected patients and HBeAg positive carriers (also known as "infectious carriers"); (c) perinatal exposure to infectious HBV-carrier mothers; and (d) in orthotopic liver transplantation patients with end stage HBV disease, pre- and post-transplant. Trials have been carried out to test the efficacy of HBIG administration in such situations (Seeff *et al.*, 1978; Redeker *et al.*, 1975; Beasley *et al.*, 1983). These studies showed that for HBIG to be effective the timing of administration was important – it should be administered as soon as possible after exposure.

1.11.3: S mutations gene associated with active and passive immunisation

The development of anti-HBs after acute or chronic HBV infection is usually associated with a recovery and a good prognosis. As discussed more extensively previously (section 1.8.2) HBsAg contains the major neutralising epitope of HBV, the "a" determinant. Anti-HBs found in convalescent and post immunisation serum binds

primarily to this determinant, which has an as yet undefined structure. It is known that the "a" determinant is comprised of conformationally dependant epitopes, as denaturation of this area by alkylation or reduction gives rise to HBsAg particles with greatly reduced antigenicity (Imai *et al.*, 1974). One proposed structure for the "a" determinant is that of a double loop with disulphide bridges between cysteines that maintain the native conformation (figure 1.7). Epitope studies show that anti-HBs binds predominantly to the second loop (Howard *et al.*, 1984). However, antibodies that bind the first loop are influenced by amino acid changes in the second loop (Waters *et al.*, 1992), implying that HBsAg-specifying epitopes are not strictly confined only to either loop but that a larger portion of the S protein contributes to the antigenic structure.

The "a" determinant is conserved though there is a degree of amino acid variation in normal isolates of HBV. However certain variants within this antigenic region have been described in association with immunisation, monoclonal/polyclonal antibody therapy as well as cases of serologically occult infection, though it is likely that in the instances of serologically occult infection, the root cause is poor HBsAg design and/or performance.

The first described HBsAg mutant was identified in an immunised baby born to an Italian carrier mother (Carman *et al.*, 1990). Despite receiving both active and passive immunisation, the baby was observed to carry both anti-HBs and HBsAg in the serum. He harboured HBV with a single point mutation that resulted in a glycine to arginine substitution at codon 145 (G145R). It has been clearly shown that yeast-derived HBsAg bearing the G145R mutation fails to bind anti-"a" antibodies or convalescent anti-sera (Waters *et al.*, 1991). In the original report (Carman *et al.*, 1990), the mutant was not detectable in the mother and presumably arose from selection pressure in the infant, and

so was considered a "vaccine escape" mutation. It is unclear if this *de novo* hypothesis of mutant selection is correct, as other studies have found putative vaccine escape S-gene mutants pre-existing in the maternal circulation, detected by sequencing of PCR derived clones (Ngui *et al.*, 1997); i.e. the mutants existed as a minor population of the viral quasipecies.

Whatever the precise nature of the origin of such mutants, the introduction of universal immunisation programs in endemic counties, though highly effective in preventing chronic HBV carriage, has led to breakthrough infections. Studies in Singapore showed that 12% of babies immunised at birth experienced breakthrough infections and that, of these, 39% showed changes in the S gene regions encoding for the MHS region of HBsAg (Zuckerman *et al.*, 1994, Oon *et al.*, 1995). The majority of these were due to the G145R amino acid substitution; though a variety of other mutations have now been described. Of children immunised in The Gambia, it was reported in one study that 8.3% had sub clinical HBV infections of which 37.5% had high levels of anti-HBs (Karthigesu *et al.*, 1994). A novel variant infection bearing a K141E mutation within the S-gene was also identified within one patient though this has not been documented since. The study concluded that such single point mutations were sufficient to induce vaccine escape in the immunised infants.

Data from several Japanese studies showed that the G145R mutant is stable (Ho *et* al 1995; Ogata *et al.*, 1997; Thakur *et al.*, 2005) and capable of horizontal transmission (Chakravarty *et al.*, 2002, Ho *et al.*, 1995, Ogata *et al.*, 1997; Thakur *et al.*, 2005), and also is capable of producing disease (Ogata *et al.*, 1997; Thakur *et al.*, 2005). Consequently HBV S-gene mutants may be considered a potential threat to public health, undermining the efficacy of vaccine programs. It has been calculated that in

those countries which introduce universal immunisation that it would take three decades after the introduction of a universal vaccination program for HBsAg mutants to be selected as the predominant variant (Wilson *et al.*, 1998, Wilson *et al.*, 2000). However, these figures are partially based on percentages from studies in Singapore and the number of mutant-carrying samples these percentages were based on is small (n = 9), therefore the margin of error in these predications is very wide; accordingly, the data should be regarded with caution (Carman, personal communication, 2004).

HBIG is commonly administered post- (and sometimes pre-) orthtopic liver transplants in attempt to prevent recurrent HBV infection after transplantation. It has been reported that S-gene mutants, most commonly, the G145R mutation, may be selected following the high doses of HBIG administered (McMahon *et al.*, 1992; Wallace and Carman, 1997). HBIG may be either monovalent or and polyclonal. In instances where the G145R mutation has been selected as a consequence of HBIG therapy patients became HBV DNA and HBsAg positive after transplantation. Mixed populations of wild type and mutant virus, including the G145R mutant circulating prior to transplant, have been identified in the quasispecies (Cariani *et al.*, 1995; Hawkins *et al.*, 1996). S-gene mutants affecting codons 144 and 145 tend to dominate, persist and be associated with worse clinical outcome in HBV re-infection (Protzer-Knolle *et al.*, 1998). More recently, antivirals are administered prior to transplantation to help reduce viral load, in conjunction with HBIG, thus the dependence on HBIG (which is relatively expensive) for transplantation is less but still necessary (Seehofer & Berg, 2005).

S-gene variants can arise naturally over the course of chronic infection without any therapeutic selection pressure, due to the natural production of anti-HBs (Hou *et al.*, 2001; Ogura *et al.*, 1999; Yamamoto *et al.*, 1994; Song *et al.*, 2005). It has been noted,

however, that in these circumstances, mutations tend to preferentially (17/18 times; 94%) occur in the 1st loop (surface amino acids 124-137) and not the second loop of the "a" determinant (Ogura *et al.*, 1999).

Vaccine-escape mutations not only prejudice therapy and undermine vaccine campaigns, they are also of diagnostic concern. This is particularly so when immunoassays based on single monoclonal antibody are used for HBsAg detection. In Papua New Guinea, for example, 5% of HBsAg-seropositive carriers would have been misdiagnosed as being seronegative using the Abbot Ausria II HBsAg radioimmunoassay (Carman, 1997). In that study, mutations at codons between 110-118 and 154-158 were particularly prevalent.

Sporadic insertion mutants in the "a" determinant have been observed in surface antigen negative hepatitis B virus Chinese carriers (Hou *et al.*, 1995). Such insertions ranged from 2 to 8 amino acids, between codons 122-124 of the S-gene. They are also had associated decreased binding to polyclonal anti-HBs, presumably due to the severe abrogation of the antigenic structure.

More recently, it has been observed that due to the overlapping nature of the HBV genome, that mutations in the S-gene may result in mutations in the RT domain of the overlapping Pol domain associated with antiviral resistance or with mutations that compensate for the lower replication fitness of the antiviral-resistant mutants (Bock *et al.*, 2002), and vice versa, as such mutations can occur in the regions of the Pol gene that overlap into the S gene and abrogate SHBs epitopes, resulting in vaccine- and diagnostic- escape variants (Torresi *et al.*, 2002b).

1.12: Treatment – Immune mediated therapy

1.12.1: Interferon

Interferons have immunomodularity, antiproliferative and antiviral effects. Lymphoblastoid and recombinant IFN-α have been used since the early 1980's in attempts to achieve sustained suppression of HBV replication and remission of HBV mediated chronic disease. IFN-α is most effective in HBeAg-seropositive and in seronegative patients with evidence of active liver disease. In such instances, long lasting clearance of HBV can be achieved in about one third of patients treated, detectable through reductions in HBV DNA levels, reductions in ALT levels and clearance of HBeAg (Hoofnagle and di Bisceglie, 1997). Typically, those who have clinical features that suggest that the patient is already in the immune clearance phase, such as high ALTs and low HBV DNA levels, respond most favourably. It has been suggested that the patient's response to interferon is also determined by many factors including ethnicity (Asians respond less well than Caucasians), sex (males perform less well than females), the genotype of infecting virus (infection with genotype B are more likely to respond than genotype C and genotype D better than genotype A) (Schaefer, 2005), homosexuality, HIV coinfection and length of chronicity (Karayiannis, 2003). The development of iatrogenic mutations in response to IFN-α has not clearly been demonstrated (Ngui *et al.*, 1999).

IFN-α use is associated with side effects, often including, flu-like symptoms, fatigue, leucopoenia and depression. Less commonly, hair loss, anorexia mood swings and irritability. IFN-α treatment may also unmask any autoimmune disorders. Recently pegylated IFN, also known as Peginteferon alpha 2a or Pegasys, was licensed for use in

the USA. It is an improved, better tolerated drug with superior bioavailability, greater ability to induce a more vigorous immune response and has been successful as a first line treatment for chronic hepatitis B infection (Janssen *et al.*, 2005). The combination of pegylated IFN plus lamivudine was shown to be only as effective as pegylated IFN alone. The same study also demonstrated significant differences in genotypes, in response rate to pegylated IFN (Janssen *et al.*, 2005).

1.13: Nucleoside analogue antiviral therapy

1.13.1: Nucleoside analogues

Antiviral agents, in particular nucleoside analogues are effective inhibitors of HBV reverse transcription and hence replication. These are chemically synthesised drugs which mimic natural nucleosides. However, unlike the natural nucleoside substrates, nucleoside analogues do not posses 3'-hydroxyl group on the deoxyribose moiety. Thus, following incorporation of a nucleoside analogue, the next incoming nucleoside cannot form the next 5'-3' phosphodiester bond needed to extend the DNA chain. Thus, when a nucleoside analogue is incorporated, nascent viral DNA synthesis (and hence viral repliacation) is halted, a process known as chain termination. Therefore nucleoside analogues are formally classified as competitive substrate inhibitors.

Nucleoside analogues can be produced in their natural D-handed conformation, or the unnatural L-handed conformation (correctly referred to as enantiomers). The HBV polymerase has a demonstrable preference for L-handed enantiomers (Davis *et al.*, 1996). Fortunately, L-handed enantiomers tend to have equal or greater antiviral activity than D-handed ones, are more metabolically stable and are less toxic (Karayiannis,

2003). Nucleoside analogues may inhibit synthesis of the (-) DNA strand by reverse transcription, synthesis of the plus DNA strand, amplification and replenishment of the cccDNA pool in the hepatocyte nucleus from non-enveloped core particles, and the formation of cccDNA in newly infected cells. Unfortunately, antivirals administered after infection have a lesser effect on the cellular cccDNA pool than they do on serum HBV DNA (Moraleda *et al.*, 1997), this places some limitations to the ultimate effectiveness of nucleoside analogue therapy as depletion of cellular cccDNA is estimated to take years due to the low rate of decay.

Many of the first nucleoside analogues used to treat patients with chronic HBV infection were initially developed or proven in HIV, given that both viruses utilise reverse transcription and there are close genotypic and phenotypic similarities between the reverse transcriptase proteins of both viruses (Toh *et al.*, 1983; Bartholemuesz *et al.*, 1998). Currently there are four nucleoside analogues licensed for use as antivirals against chronic HBV infection: lamivudine, adefovir, entecavir and most recently telbivudine. Others are undergoing clinical evaluations. A list of the currently licensed nucleoside analogues against HBV and those in development is shown in Table 1.1 below:

Drug Name	Company	Status	Analogue
Epivir-HBV (Lamivudine)	GlaxoSmithKline	FDA approved 1998	L-D citydine
Hepasera (Adefovir Dipovoxil)	Gilead	FDA approved 2002	acyclic adenosine ring
Baraclude (Entecavir)	Bristol-Myers Squibb	FDA approved 2005	guanosine analogue
Telbivudine (LdT)	Idenix	FDA approved 2006	β-L thymine
Clevudine (L-FMAU)	Pharmasset	Phase III South Korea & Phase II USA	β-L thymine
Emtrictabine (FTC)	Gilead	Phase III	L-D cytidine
Viread (Tenofovir)	Gilead	Phase III	acyclic adenosine ring
Valtorcitabine (monoval LdC)	Idenix	Phase II	L-D cytidine
Amdoxovir (DAPD)	RFS Pharm	Phase II	guanosine analogue
RCV (Racivir)	Pharmasset	Phase II	racemic mixtre (50:50) of L-D cytidine and D-D cytidine

Table 1.1 – Nucleoside analogues against HBV, licensed and in development.

(Source: http://www.hepb.org/professionals/hbf_drug_watch.htm).

1.13.2: The development of viral resistance to nucleoside analogues

Resistance to antivirals is typically first observed as 'phenotypic resistance' - a return in the patient of virus, combined with rising ALT levels. Viral genotypic resistance may be confirmed following sequencing the reverse transcriptase domain of the viral Pol gene, or through the use of a SNP detection assay.

The lack of proof reading by the HBV Pol during HBV replication results in a high spontaneous mutation rate of approximately $1x10^{-5}$ to $3x10^{-5}$ nucleotide substitutions per site per year may occur (Locarnini, 2004). HBV is extremely prolific, producing up to 10^{10}-10^{11} virions on a daily basis. Therefore, a large number of mutations can be potentially produced at every site on every genome (Nowak *et al.*, 1996; Zeuzem *et al.*, 1997). While the mutation rate of HBV is high in comparison to other DNA viruses, the overlapping nature of ORFs places great constraints against the development of spontaneous mutants. The mutation rate for HIV (which also uses-error prone reverse transcription) is 100-1000 fold higher (Roberts *et al.*, 1988). Despite the relative degree of genomic constraint against mutation in HBV, resistance to all currently licensed antivirals has been observed (Zoulim, 2004).

1.13.3: Lamivudine

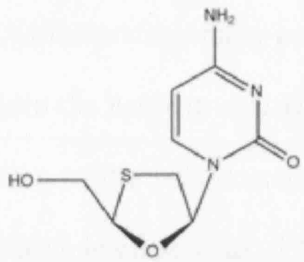


Figure 1.11 – Molecular structure of Lamivudine

Lamivudine (-)-β-L-2', 3'-dideoxy-3'thiacitydine (figure 1.11), is an orally administered nucleoside analogue of deoxycytidine and structurally is a negative L-handed enantiomer. Lamivudine efficiently suppresses HBV replication in HBeAg sero-positive and sero-negative chronic carriers. Viral suppression is transient and withdrawl of therapy sees the rerun of virus levels. Lamivudine is well tolerated by patients and can lead to clear clinical outcomes, such as decreased ALTs, loss of HBeAg, seroconversion to anti-HBe positive status, reductions in serum viral HBV DNA and improvement in liver histology. Prolonged use is associated with the emergence of drug-resistant mutants. Resistance may be found in about 14%, 38%, 54%, 66% and 70% of treated HBeAg positive and negative patients after 1, 2, 3, 4 and 5 years of therapy, respectively (Leung *et al.*, 2001; Liaw *et al.*, 1999; Kim *et al.*, 2001; Chang *et al.*, 2000; Wright, 2004). Resistance is typically associated with mutations in the tyrosine-methionine-aspartate-aspartate (YMDD) motif of the C sub domain of the HBV RT. The resistant phenotype is due to genotypic changes that result in substitution of methionine to either valine or isoleucine at codon rt204 (according to the nomenclature outlined by Stuyver *et al.*, 2001). These HBV rtM204V/rtM204I

mutations are analogous to the well-characterised HIV rtM184V lamivudine-resistance mutation, which appears some weeks after the introduction of monotherapy. The mechanism for the HIV resistance mutation in HIV infected patients is due to steric hindrance from amino acid substitutions in the YMDD motif which prevents lamivudine binding in the dNTP pocket whilst preferentially incorporating natural dNTPs; hence no lamivudine is incorporated into the nascent viral DNA chain, and no chain termination occurs (Gao *et al.*, 2000). Due to the well-documented similarities between HIV and HBV RT (Toh *et al.*, 1983; Bartholemuesz *et al.*, 1998), the mechanism of resistance for HBV is thought to be the same as for HIV.

In HBV, the appearance of methionine substitutions to either valine or isoleucine at the YMDD motif has been shown *in vitro* to increase the IC_{50} by 1000 to 10,000 fold (Allen *et al.*, 1998). Thus, an increase in lamivudine dose cannot overcome resistance. Though initial studies suggested that the rtM204V mutation was most common, it is now thought that the appearance of this mutation may be genotype dependant rtM204V being more associated with genotype A and rtM204I with genotype D, (Hadziyannis *et al.*, 2000).

Compensatory mutations have been identified *in vitro* that frequently occur in conjunction with mutations in the YMDD motif, as the YIDD and YVDD mutants are considered replication defective (Melegari *et al.*, 1998; Chayama *et al.*, 1998; Ono-Nita *et al.*, 1999; Fu *et al.*, 1998). The most commonly compensatory mutation identified is the rtL180M mutation. It has been well characterised as being able to aid the functionally less fit YVDD mutation, with which it is very often associated, by partially restoring replicative fitness (Ling and Harrison, 1999). More recently, the rtV173L mutation was identified. In one study, this has been found in about a quarter of patients

who carried the YVDD mutant; it has been characterised to partially restore replication fitness (Ogata *et al.*, 1999; Delaney *et al* 2003). This mutant may be associated with aggressive liver disease, but other studies contradict this finding (Bock *et al.*, 2002). The mutation L80I in the F domain is a further possible compensatory mutation (Ogata *et al.*, 1999).

That YMDD mutants lack fitness is confirmed by the observation that when lamivudine is removed, the resistant mutant virus population recedes and the wild type virus population returns (Buti *et al.,* 1997; Chayama *et al.*, 1998; Lau *et al.* 2000; Da Silva *et al.*, 2001). Lamivudine resistant mutants may, however, remain detectable for many months after stopping treatment, and be rapidly selected upon re-exposure to the drug (Lok *et al.*, 2002). It is apparent that such mutants retain a propensity to induce disease like their wild type counterparts (Ayres *et al.*, 2003).

1.13.4: Adefovir Dipovoxil

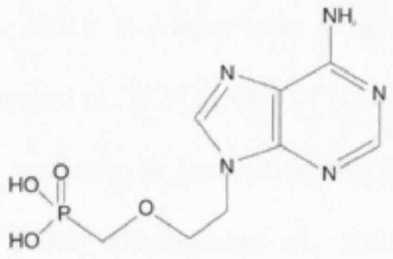


Figure 1.12 – Molecular structure of Adefovir Dipivoxil

Until recently, interferon alpha and lamivudine were the only drugs approved for the treatment of chronic hepatitis B infection. Adefovir dipivoxil (9-[2-(phosphonylmethoxy) ethyl] adenine; PMEA; GS 393) (figure 1.12) has recently been licensed for use in the treatment of chronic hepatitis B infection in adults in both the USA and Europe. It has a broad spectrum of activity against hepadnaviruses and retroviruses (De Clerq *et al.*, 1986). An orally bio-available prodrug of adefovir, adefovir dipivoxil is a nucleotide analogue of deoxyadenosine monophosphate with an acyclic ring structure - (9-[2-phosphonomethoxy] ethyl)-adenine. It has an extremely favourable drug resistance profile: a study of 39 HBeAg positive and negative patients treated with ADV for up to 60 weeks showed no aa substitutions that could be linked to a resistance phenotype or loss of DNA suppression in vivo (Yang *et al.*, 2002). Further phase III studies involving much larger patient cohorts have corroborated this observation: in 695 patients, no adefovir-resistant mutations were identified after 48 weeks of therapy; the drug was well tolerated and effectively suppressed HBV replication in both HBeAg seropositive and HBeAg seronegative chronic carriers (Marcellin *et al* 2003, Hadziyannis *et al* 2003) regardless of genotype (Westland *et al*

2003). Critically, adefovir has been demonstrated to have an antiviral activity versus lamivudine-resistant mutants (Xiong *et al* 1998., Ono-Nita *et al.*, 1999, Perrio *et al.*, 2000 and Benhamou *et al.*, 2001). A longer-term study identified a novel adefovir mutation, rtN236T which emerged in 2/124 (1.6%) of HBeAg seropositive patients after 96 weeks of therapy. This mutation is located in the D sub domain of the HBV polymerase (Angus *et al.*, 2003, Villneuve *et al.*, 2003). More recently, adefovir resistance was observed in 5.9 % of patients after 144 weeks of therapy: the N236T mutant was detected at a rate of 3.6% and a novel A181V mutant at a rate of 1.8%. (Hadziyannis *et al.*, 2005). The N236T mutation decreases sensitivity to Adefovir 13-fold and it has been suggested that N236T favours binding to the natural dATP substrate (Yang *et al.*, 2004). The rtA181V mutation decreases sensitivity to adefovir by 2-3 fold. The adefovir resistance mutation N236T has been analysed for cross-resistance to other antivirals, but has been found to exhibit sensitivity to lamivudine, entecavir, telbivudine and emtricitabine (Yang *et al.*, 2004).

It is still unclear as to why the resistance rate for adefovir is comparatively low compared to lamivudine, though various mechanisms have been postulated (Zoulim, 2002; Westland *et al.*, 2003). The adefovir molecule is very similar to dATP, thus limiting steric discrimination by the HBV Pol, which underlies lamivudine resistance (Gao *et al.*, 2000). Moreover, the flexible molecular structure of adefovir may ease binding into the dNTP-binding pocket of reverse transcriptase, despite the presence of a resistance mutation.

While adefovir has a much better resistance profile - 1.6% resistance over two years (Yang *et al.*, 2002) compared to 38% for lamivudine (Liaw *et al.*, 1999) - it is nephrotoxic. Nephrotoxicity was noted in studies to monitor the ability of ADV to

suppress HIV replication in a study in which HIV-infected patients received high doses of the drug - around 130 mg a day (Fischer *et al.*, 2001). In the Gilead Phase III clinical trial for the use of adefovir in HBeAg seropositive and HBeAg seronegative HBV infection, the lower dose of 30 mg a day could be found to be associated with nephrotoxicity (Marcellin *et al.*, 2003, Hadziyannis *et al.*, 2003). Such studies have led to the recommendation that a dose of not more than 10 mg a day be used for chronic hepatitis B. As the N236T and A181V mutations decrease sensitivity to adefovir by a factor of 10-13 and 2-3 respectively, an increase in the drug dose to combat resistance would be unsafe, since the dose required to inhibit viral replication potentially leads to toxicity. So adefovir (or any other drugs licensed for HBV therapy) may enjoy a better resistance profile and a better viral suppression profile than lamivudine, though lamivudine is still considered amongst the most safe of anti-HBV antivirals.

1.13.5: Entecavir

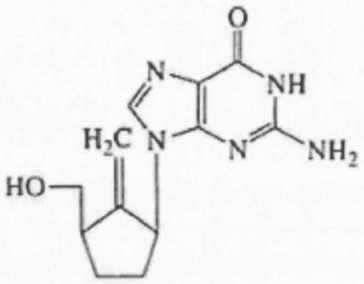


Figure 1.13 – Molecular structure of Entecavir

Entecavir - 2-amino-1,9dihydro-9-[(1S,3R,4S)-4-hydroxy-3-(hydroxymethyl)-2 methylenecyclopentyl]-6H-purin-6-one monohydrate (figure 1.13)- is a deoxyguanosine analogue that selectively inhibits HBV polymerase as well as the priming step of HBV replication. In Phase II clinical trials entecavir demonstrated that it was superior to lamivudine in suppressing HBV replication (Lai *et al.*, 2001). It is now known to be the most potent, licensed inhibitor of HBV replication. It is also a safe drug, which was well tolerated in safety trials, with no significant ill effects in comparison to placebo (de Man *et al.*, 2001).

Resistance to entecavir was reported in 2 patients who received entecavir in combination with lamivudine for 80-100 weeks (Tenney *et al.*, 2004). Several novel mutations were noted: rtI169T/sF161L, rtT184S/sL176V, rtS202I/sV194F and rtM250V (Tenney *et al.*, 2004). Thus far, resistance to entecavir has only been confirmed in *in vitro* studies, but these mutations occurred in conjunction with pre-existing lamivudine mutations (Tenney *et al.*, 2004). An rtT184L mutation has also been observed in one patient, who experienced breakthrough after 2 yr of lamivudine + entecavir combination

therapy (Fung and Lok, 2003). Entecavir is, however, active against both lamivudine- and adefovir-resistant HBV (Shaw and Locarnini, 2005), and in conjunction with its potent effects, will become an important addition to the antivirals armamentarium.

1.13.6: Combination therapy

The advantage of combination therapy is its potential to reduce the risk of drug resistance emerging in comparison with monotherapy. Moreover, the use two or more drugs taken simultaneously can achieve additive or synergistic effects. Synergism is more likely if the two drugs being administered have different modes of action, such as underlies the combinations used for high active antiretroviral therapy (HAART) in HIV infection. However, unlike HIV, in which there are a variety of agents able to interfere with various stages of the viral life cycle, only nucleoside analogues and interferon are available for combination therapy for HBV. Indeed, several studies have shown that a combination of lamivudine and IFN-α can lead to lower rates of resistance (Barbaro *et al.*, 2001; Mutimer *et al.*, 2000a; Sanantonio *et al.*, 2002; Schalm *et al.*, 2000).

In a study comparing lamivudine monotherapy to an adefovir + lamivudine combination, resistance at one year was detected at the rates of 2% for adefovir + lamivudine compared to 20% for lamivudine alone (Fung and Lok, 2003). The adefovir + lamivudine resistance rate is not an improvement to that observed for adefovir alone (Angus *et al.*, 2003, Villneuve *et al.*, 2003). To date no synergism has been identified; there is no clear evidence of higher rates of HBeAg seroconversion, histological improvements or steeper decreases in serum HBV DNA levels. However, longer-term studies into combination therapy should reveal if this lower resistance rate translates to reductions in HBV DNA levels in comparison to monotherapy.

Other combinations have also proven to be ineffective, or less effective than monotherapy. E.g., when Lamivudine + telbivudine therapy was compared to therapy with telbivudine alone, the resistance rates were 10% and 5%, respectively (Fung and Lok, 2003). HBeAg seroconversion rates were actually lower in the combination therapy group than for the monotherapy group. With 4 licensed antivirals and pegylated interferon now available, and more antivirals in the pipeline, an increasingly complex picture is emerging to reveal antiviral resistance in HBV.

Sequential monotherapy has not been advocated as it may result in multi-drug resistance (Mutimer *et al.*, 2000b). In the absence of drugs that would promote synergism, it seems likely that clinicians will use monotherapy and a second drug 'on top' when resistance emerges. There is concern that even the use of two similar classes of drugs in combination (e.g. nucleoside analogues) could still lead to resistance in the long term thereby fostering the emergence of multi-drug resistant mutants. A recent *in vitro* study showed that a triple mutant, sporting the rt204V + rtL180M lamivudine resistance mutations and the rtN236T adefovir resistance mutation, is still susceptible to interferon and entecavir (Brunelle *et al.*, 2005). The cross resistance of certain reverse transcriptase mutations against current antivirals is summarised Table 1.2 below.

Mutant	Lamivudine	Telbivudine	Entecavir	Adefovir	Tenofovir
rtV173L	INTERMED.				
rtL180M	INTERMED.	INTERMED.	LOW		
rtA181T/V	INTERMED.			HIGH	
rtT184S			HIGH		
rtA194T					HIGH
rtS202I			HIGH		
rtM204V/I/S	HIGH	HIGH	LOW		
rtN236T				HIGH	

Table 1.2 – Cross resistance of nucleoside analogues against HBV. HIGH = Well defined mutation with high IC_{50}. INTERMED (Intermediate) = Well defined resistance compensatory mutation leading to high IC_{50} in conjunction with other mutations OR mutation leading to moderate increase in IC_{50}. LOW = Minor and less well studied resistance compensatory mutation (Liaw, 2005).

1.13.7: Significance of S and P gene overlap in antiviral resistance

The HBV Pol has not been crystallised, and as such its molecular structure has not as yet been characterised by X-ray crystallography diffraction. However, a recent *in silico* generated model of HBV polymerase has been created by comparison of conserved motifs in HBV reverse transcriptase to the protein crystalline structures of MuLV and HIV (Das *et al.*, 2001) RTs. This work has been of value in inferring HBV RT structure. All nucleic acid replication polymerases work in the same manner, whether they come from prokaryotes, eukaryotes or viruses, and whether they replicate DNA or RNA from a DNA or RNA template (Delaney *et al.*, 2001). The basic structure is conserved in a shape approximately that of a hand with the protein domains termed as the palm, fingers and thumb. Each part of the hand has a different role in DNA

replication. The palm is the site for nucleotide binding; the conserved HBV reverse transcriptase sub domain motifs B, C and D roughly sit in the palm (figures 1.8 and 1.9). As nucleotide analogues mimic nucleotides, mutations against them most commonly arise in the palm domain (HBV RT motifs B, C and D) in variants that exhibit drug resistance. The thumb domain has a role in template stability (and is equivalent to HBV RT motifs F G and A), and finally the fingers domain has a role in primer binding and in nucleotide binding (and is roughly equivalent to motif E). It is of note that the adefovir resistance mutation N236T occurs in a predicted fingers domain of HBV RT, confirming that such regions may influence resistance. Mutations in the fingers domain might alter the interaction of unnatural nucleoside analogues, leading to steric discrimination. Virus harbouring such mutations would continue to replicate with natural cellular nucleotides with no nascent DNA chain termination or cessation of replication (a phenomena known as substrate selection) as would be seen in wild type virus that encountered nucleoside analogues. The *in silico* generated model (Das *et al.*, 2001) is significant because it shows a second putative fingers domain for HBV polymerase RT at codons rt112-167. These data, in conjunction with that observed in a study of HBeAg-seronegative patients on lamivudine are significant, as 8/10 patients in the study developed virus bearing classic YMDD resistant genotypes, but the mutant virus also developed mutations upstream of the YMDD motif in the HBV polymerase RT gene at the time that the resistant genotype emerged (Lok *et al.*, 2000). The majority of these upstream mutations clearly fell into the newly identified fingers domain (rt112-167). Furthermore, in 2 out of the 8 patients who developed YMDD mutations, upstream mutations rtN122L, rtQ130P and rtA200V overlapped into the S gene, causing the mutational changes sT114S, sK122R and sL192F respectively.

Looking back, a number of earlier studies have also identified mutations in the HBV polymerase RT which are not typical substitutions expected in the YMDD motif or the typical sites of compensatory mutations. Again, these mutations arose during lamivudine therapy and overlap the S gene leading to mutational change in the S gene reading frame (Tipples *et al.*, 1996; Bartholomew *et al.*, 1997; Terrault *et al.*, 1998; Ogura *et al.*, 1999; Shields *et al.*, 1999; Lok *et al.*, 2000). These described 'fingers' mutations that overlap into S may still be compensatory mutations (despite the current lack of functional evidence) by altering reverse transcriptase behaviour of the mutants. It is possible to postulate that these overlap mutations are essentially driven by selective pressure on the RT protein alone due to the strong selective pressure of nucleoside analogue therapy, and that overlap into the S gene is perhaps coincidental. Antivirals may select a mutational change in the SHBs that confers advantage to the virus. Figure 1.14 below (Torresi *et al.*, 2002b) shows in detail how these upstream *Pol* mutations impact upon the SHBs.

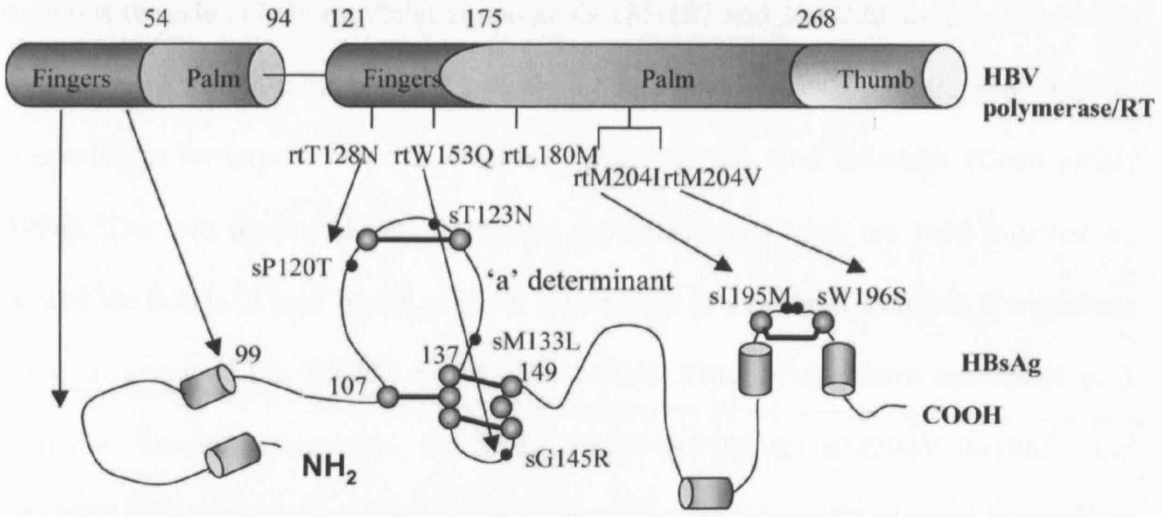


Figure 1.14 - Lamivudine selected mutations in HBV reverse transcriptase can trigger mutational change in HBsAg. (Torresi *et al.*, 2002b). The SHBs structural model used for comparison is that of Chen *et al.*, 1996 while the polymerase model is that of Das *et al.*, 2001.

Mutations of the putative second fingers domain overlap directly into the region that encodes for the major hydrophilic region of the HBsAg and often the "a" determinant itself. Thus although such mutations are essentially mutations of the HBV RT, they have the potential to alter the MHR and the "a" determinant, possibly affecting its conformation and hence its interaction with antibody directed to it. Thus drug resistance may lead to some degree of vaccine resistance.

Classic resistance mutations at the YMDD motif in HBV RT can also lead to mutational change in S gene. According to one model for SHBs (Chen *et al.*, 1996), amino acids 194 and 195 could be significant as this region of HBsAg (the carboxyl terminal end)

contains two alpha helices whilst amino acids 183-187 and 196-220 are predicted to be hydrophobic. The regions between containing the alpha helices are hydrophilic and are presumed to be exposed on the external surface of the viral envelope (Chen *et al.*, 1996). The two alpha helices, which are separated by a turn, are held together by disulphide bonds. These bonds may be susceptible to structural changes if mutations were to occur at the YMDD motif. The classic YMDD mutations can result in 3 possible mutations in the S ORF: rtM204Is/I195M, rtM204V/sM196L and rtM204V/sM196S. Such amino acid substitutions in the externally exposed hydrophilic structure in amino acids s187-196 might result in the development of secondary structural changes within this structure. The exposed amino acids s187-196 may be part of a discontinuous epitope and may interact with downstream regions of the S protein hence acting as B-cell epitope to a greater degree than is currently suspected. Alternatively, the 187-196 region may act as an epitope 'moderator': it has been shown that mutations in HBsAg may affect distant epitopes (Waters *et al.*, 1992). Even if there were no direct or indirect interactions of amino acids s187-196 amino acids with the immunodominant upstream amino acids, these may, given their exposed nature, play some minor role as B-cell epitopes. It is then possible to hypothesise that mutational change in the YMDD motif may also result in reduced binding of antibody to virus, as well as mutations in the putative second fingers domain.

Owing to the overlapping nature of the HBV genome there is significant constraint on sequence divergence when driven by selective pressures. Thus SHBs mutations may be generated by mutations driven by the selective pressure of antiviral therapy resulting either with classical resistance substitutions in the YMDD motif or those in the 'fingers' domain.

Such a phenomenon has already been the subject of one study (Torresi *et al.*, 2002b) which examined 2 fingers mutations and 2 mutations in the YMDD motif. That study sought to examine how these changes in HBsAg, generated via site-directed mutagenesis and expressed in recombinant yeast, affected binding to pooled vaccinee sera. The results showed that mutations of the YMDD motif could result in a slightly reduced binding to anti-HBs; moreover, the mutations in the putative fingers domain and the compensatory mutations were associated with greatly reduced binding of HBsAg to anti-HBs. Certain combinations of mutations (a YMDD substitution rtM204V/sI195M and a fingers domain mutation rtV173L/sE164D) demonstrated a high degree of binding reduction to pooled vaccine sera comparable to the classical vaccine escape mutant G145R. Further work by the same group (Torresi *et al.*, 2002a) went on to show that some of these fingers mutations - rtT128N, rtQ130P, rtW153Q and rtG153E - could restore the full replication phenotype of virus with mutations in the YMDD motif, thereby confirming their compensatory status. Mutations driven by drug resistance, could therefore lead to vaccine escape.

It should be noted that it is also possible to consider the same S/P gene overlap issue but in reverse. For example, the HBsAg mutation sP120T, a recognised vaccine escape mutant, is equivalent to rtT128N, a recognised lamivudine compensatory mutation (Bock *et al.*, 2002). Thus, a mutation driven by selection in the S gene may result in a mutation in HBV RT and thus potentially generate a phenotype resistant to lamivudine, or other nucleoside analogue. Some studies which found mutations of both genes, have led to uncertainty as to whether the mutational changes observed arose as a consequence of nucleoside analogue therapy or anti-HBs selective pressure (Lee *et al.*, 2005).

In summary, mutations in the S or P gene potentially affect the efficacy of vaccine prophylaxis, passive immunisation and antiviral chemotherapy. More antivirals are becoming available for treatment of chronic HBV. The adefovir resistance mutation rtA181V/sW172stop and the entecavir resistance mutations rtI169T/sF161L, rtT184S/sL176V, rtS202I/sV194F all affect the S/P gene overlap. HBIG, still used in some clinical circumstances, and hepatitis B vaccine, widely administered share the propensity to induce mutational change in the MHR which affects HBsAg binding to anti-HBs. Thus the emergence of antiviral resistance in individuals may be a 'back door' to the generation of vaccine and diagnostic escape mutants, potentially affecting the viral interaction with the patient's own anti-HBs response (whether natural or the consequence of immunisation) or therapeutically administered anti-HBs. Moreover, vaccine escape mutations may, in turn, stimulate mutational change in RT motifs, stimulate resistance or resistance compensatory mutations, and prejudice the success of nucleoside analogue therapy.

Such considerations raise public health concerns. Although transmission of lamivudine induced-mutants is rare, its occurrence has been documented (Thibault *et al.*, 2003). Some antiviral resistant variants arise as a natural consequence of untreated chronic infection, (Kirishima *et al.*, 2002: Kobayashi *et al.*, 200; Shin *et al.*, 2003; Heo *et al.*, 2004; Leon *et al.*, 2004). Crucially, even in countries which implement no universal immunisation policy, but whose healthcare systems do have the resources to administer antiviral therapy, vaccine escape-mutations may be selected by this route. Similarly, vaccine-induced pressure in highly endemic countries with immunisation programs resulting in vaccine escape mutants may be problematic as the presence of such mutants in a patient may prejudice their response to antiviral therapy (Paik *et al.*, 2001).

1.14: Aims of Study

Antiviral resistance mutations can adversely affect the antigenicity of HBsAg (Torresi *et al.*, 2002b). The mechanism by which this happens is, as yet, unclear. Although it is known that the "a" determinant of HBsAg is the immunodominant region (Howard *et al.*, 1984; Zheng *et al.*, 2004), numerous studies have now suggested that flanking regions in the MHR may affect antigenicity (Wallace *et al.*, 1994; Carman *et al.*, 1997; Terrault *et al.*, 1998; Chong-Jin *et al.*, 1999; Kfoury-Baz *et al.*, 2001; Jeantet *et al.*, 2004; Wagner *et al.*, 2004). Moreover, current structural models predict a more complex structure for this region, and propositions of discontinuous epitopes and potentially novel epitopes have been made (Waters *et al.*, 1992; Steward *et al.*, 1993; Prange and Streek, 1995; Chen *et al.*, 1996; Paulij *et al.*, 1999). Other studies have observed the emergence of mutations in the MHR of HBsAg due to antiviral resistance mutations (Tipples *et al.*, 1996; Bartholomew *et al.*, 1997; Terrault *et al.*, 1998; Ogura *et al.*, 1999; Shields *et al.*, 1999; Lok *et al.*, 2000). Finally, mutations that confer antiviral resistance may arise naturally over the course of an untreated chronic infection (Kobayashi *et al.*, 2001; Shin *et al.*, 2003; Kirishima *et al.*, 2002; Heo *et al.*, 2004; Leon *et al.*, 2004). Thus there is a growing appreciation of the potential of antiviral therapy to select vaccine resistant or serologically undetectable mutants, but yet little is known about their impact upon HBsAg epitopes.

The first aim of the study is to study how nucleoside analogue-induced mutations (specifically those currently licensed – lamivudine, adefovir and entecavir) at the YMDD motif, fingers domain and other regions of HBV polymerase might affect the antigenic properties of HBsAg. Mutant HBsAg will be generated through site directed mutagenesis by the transfection of eukaryotic cells, with an expression vector

containing the SHBs gene. Enzyme-linked immunoassays (ELISAs) using a panel of monoclonal antibodies, which bind distinct regions of the "a" determinant will be employed to allow an indication of how epitopes within the "a" determinant are perturbed by single or multiple drug resistant mutations in the overlapping P gene. Such an approach should permit the effect of various nucleoside analogue-induced mutations on discrete epitopes to be monitored. By specifically probing the "a" determinant exclusively, it may be possible to determine if reductions in antigenicity with drug resistant mutants observed by some studies using pooled vaccinnee anti-HBs (Torresi *et al.*, 2002b) are due to a specific interaction with the "a" determinant, or if other epitopes are implicated. The use of polyclonal anti-HBs to assay mutant HBsAg would give an indication if any changes in antigenicity in mutant HBsAg observed. Moreover, the contrast between monoclonal data and polyclonal anti-HBs data will allow inferences to made about whether YMDD mutations affect putative epitopes outside the "a" determinant as has been proposed by other studies (Torresi *et al.*, 2002b).

The full emergence of vaccine escape mutants is estimated to take decades (Wilson *et al.*, 2000). Lamivudine-resistant mutants of HBV are known to be less replication fit or competent, although their transmission has been documented rarely (Thibault *et al.*, 2003). Given use of HBIG, vaccine and antivirals in the UK there also exists the potential for drug resistance mutants to induce vaccine resistant mutants and vice versa. From a public health perspective, a survey of the prevalence of vaccine-escape and nucleoside analogue-resistant mutants requires to be taken before hepatitis B immunisation and nucleoside analogue therapy become more widespread, in order that the background prevalence of the mutants that might be naturally occurring can be ascertained. Such an action would allow the rise in prevalence of true iatrogenic mutants (whether arising from vaccination or antiviral therapy) to be monitored over time. Such

an enhanced surveillance strategy would allow an assessment of the impact of iatrogenic mutations on circulating virus. Accordingly, to create the foundation for future surveillance of iatrogenically-induced HBV mutants, a second arm of the study was devised to study the molecular epidemiology of patients with incident hepatitis B. Immunisation in the UK is limited to certain risk groups such as healthcare workers and others. By correlating molecular data with risk factors it could be possible to observe for trends associated with the emergence of any mutants.

CHAPTER 2

MATERIALS AND METHODS

2.1: General Methods used in this study

2.1.1: DNA quantification

DNA (10µl) was diluted in 1/100 distilled water and analysed spectrophotometrically at wavelengths of 260 nm and 280 nm using a UV 300 spectrometer (Unicam). The concentration of DNA was then determined. One absorbance unit (A_{260}) was taken to be equivalent to 50 µg/ml of double stranded DNA, as follows:

DNA concentration in µg/ml = 50 x Abs_{260} x dilution factor

Purity of DNA was assessed by obtaining the relative absorbances at 260 nm and 280 nm. Proteins, which absorb more heavily around 280 nm (primarily due to tryptophan absorbance, with a lesser contribution from tyrosine residues), will shift the A_{260}/A_{280} ratio lower if present as contaminants in a DNA sample. Accordingly, the A_{260}/A_{280} ratio was normally 1.8 to 2.0 indicating low levels of protein contamination.

2.1.2: Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed in either 1% or 2% agarose (Severn Biotech) containing 0.005 % ethidium bromide, using TAE buffer (40 mM Tris HCL, 20 mM sodium acetate 2.5 mM EDTA, pH 8.0). For low melting point (LMP) gels, Low Melting Point Agarose was used (Gibco BRL). Samples were mixed with 6 X loading buffer (0.25% bromophenol blue, 30% glycerol) before loading onto the gel. Gels were run at 50-100 V until the dye front had moved to approximately 1 cm from the bottom of the gel. Gels were visualised and photographed using the Epi Chemi II Darkroom and Labworks image acquisition and analysis software (UVP).

1 µl of hyper ladder 1 (Bioline) quantification ladder was run alongside the samples as a gel control, each band in the ladder representing an exact amount in ng of double stranded DNA. Once the gels had undergone electrophoresis and visualised by UV transillumination, it was thus possible to compare the intensity of sample DNA to that of the DNA ladder and so roughly estimate DNA quantity in ng/µl to the nearest 20 ng/µl. For precise DNA quantity data, DNA absorbance in solution was measured at 260 nm (see section 2.1.1).

2.1.3: Purification of DNA

DNA was purified from solution or excised LMP agarose gel bands using the Promega 'Wizard PCR preps' DNA purification kit, applying the vacuum manifold protocol solution method, according to the manufacturer's instructions. For LMP agarose slices, the slice was melted by heating to 70°C; then a resin (1 ml) was added and mixed for 20 s. In the case of solution purification 100 µl of Direct Purification Buffer was added and mixed by vortexing, after which the resin (1 ml) was added and vortexed for 20 s. Thereafter, the protocol applied for gel and solution purification was the same, resin and DNA being added to a kit mini-column attached to a vacuum manifold. The column was washed under vacuum with 2 ml isopropanol and dried for 30 s. This was dried more by centrifuging at 10,000 g for 2 min to remove the isopropanol. The mini-column was applied to a clean micro centrifuge tube and DNA was eluted by the addition of 50 µl nuclease free water (Promega); this was incubated at room temperature for 1 min and then centrifuged at 10,000g for 1 min. DNA was either used directly in following reactions or stored at -20°C for later use.

2.1.4: Transformation of One Shot™ INVαF'/Top10 Chemically Competent Cells

A single vial (50 µl) of One Shot™ INVαF'/Top10 chemically competent cells (Invitrogen) was thawed on ice for each transformation. 100 ng plasmid DNA or ligation mixture was added to the cells and gently mixed by tapping. Vials were then incubated on ice for 30 min, shock-heated at 42°C for exactly 30 s and then quenched on ice for 2 min. 250 µl SOC media, which had been pre-warmed to 37°C, was added, and the vials were then incubated horizontally for 1 hr at 37°C with shaking at 225 rpm. The transformation mixture was spread on LB Agar plates containing kanamycin (50 ng/µl) and incubated overnight at 37°C. Individual colonies were then picked for further characterisation.

2.1.5: Plasmid isolation and purification – Qiagen miniprep

Qiagen minipreps allowed the isolation of small amounts of high quality purified plasmid DNA from cloned bacterial cells for use in downstream applications such as subcloning and sequencing. The system is based on the alkaline lysis of bacterial cells followed by the adsorption of DNA onto silica gel following which the DNA can be washed then eluted. Typically, bacterial cells from 3 ml of overnight culture in Luria-Bertani (LB) broth containing 50 µg/ml kanamycin were pelleted at 10,000 g for 5 min. The cells were resuspended in 250 µl Buffer P1 (50 mM Tris HCL, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A) to which 250 µl buffer P2 (20 mM NaOH, 10% SDS) was added. This was then inverted 6 times to mix gently; 350 µl Buffer N3 (3 M potassium acetate, pH 5.5) was added with immediate mixing of the tube to prevent precipitation. After centrifuging for 10 min at 10,000 g the supernatant was added to a spin column. This was inserted into a vacuum manifold (Promega) and a vacuum applied. The column was washed by the addition of 500 µl buffer PB followed by 750 µl buffer PE. Residual buffers were removed by further centrifugation at 10,000 g for 1 min. The

column was then added to a micro centrifuge tube, allowing DNA to be eluted by adding 50 µl nuclease-free water (Promega), incubation at room temperature for 1 min and centrifugation at 1 min at 10,000 g. Resulting DNA could then be used directly in downstream applications or stored for further use at -20°C.

2.1.6: Plasmid isolation and purification – Qiagen Qiafilter endo free maxiprep

Large amounts of highly purified endotoxin-free plasmid DNA suitable for eukaryotic cell transfection was prepared using the Qiagen 'Qiafilter Endo Free Maxiprep' kit. The kit uses a modified version of the alkaline lysis method followed by binding of plasmid DNA to an anion exchange resin. RNA, proteins and low molecular weight impurities are removed by a medium salt wash, and plasmid DNA is eluted in a high salt buffer. The DNA is then pelleted and desalted using isopropanol precipitation. This procedure includes an endotoxin removal stage, critical for effective transfection of DNA into mammalian cells, where DNA is incubated with an 'endotoxin removal' (ER) buffer (composition not provided by manufacturer) which prevents LPS from binding to the anion exchange resin during DNA purification. The detailed procedure is as follows: a single colony is picked from a freshly streaked plate and used to inoculate a starter culture of 5 ml LB containing kanamycin (50 ng/µl) as the selective antibiotic. The starter culture is grown at 37°C, 225 rpm for 8 h then diluted 1/1000 in fresh selective LB medium and incubated overnight (c. 16 h) at 37°C, 225 rpm. Cells are harvested by centrifugation at 6,000 *g* for 15 min at 4°C. The bacterial pellet is resuspended 10 ml buffer P1 (50 nM Tris HCL, pH 8.0, 10 mM EDTA, 100 µg/ml RNAase A) to which 10 ml buffer P2 (200nM NaOH, 10% SDS) is added. Tubes are inverted gently 4-6 times and incubated at room temperature for 5 min to allow cells lysis to occur. To the lysate, 10 ml buffer P3 (3 M sodium acetate, pH 5.5) is added for neutralisation. The lysate is filtered using a QiaFilter to remove precipitated proteins, genomic DNA and detergent.

The filtrate is then treated with ER buffer for 30 min on ice to remove endotoxins from the DNA. The lysate is applied to an anion exchange column (previously equilibrated with buffer QBT [750 mM NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol, 0.15 % Triton X-100]) and allowed to enter the resin by gravity flow. The column is washed with 60 ml buffer QC (1.0 M NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol), and DNA eluted into an endotoxin-free tube by the addition of 15 ml buffer QN (1.6 M NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol). DNA is precipitated by the addition of 10.5 ml isopropanol, mixed and centrifuged at 15,000 g for 30 min at 4°C. The DNA pellet is then washed with 5 ml of endotoxin-free 70% ethanol and centrifuged at 15,000 *g* for 10 min at 4°C. The pellet is air dried, and resuspended in TE buffer (10mM Tris HCL, pH 8.0, 1 mM EDTA), following which DNA was quantified using spectrophotometry (section 2.1) and stored at 20 °C.

2.1.7: DNA sequencing

All samples were sequenced using the Beckman CEQ2000 automated capillary array sequencer (Beckman Coulter, California, USA). Purified DNA was added to the sequencing reaction consisting of the following components: 100 µg/µl purified DNA (*x* µl), 2 µl sequencing primers (3 pmol/µl), 8 µl CS Quick Start sequencing kit mix (Beckman) and nuclease-free water to a total volume of 20 µl. Sequencing PCR reactions were purified by ethanol precipitation. The 20 µl sequencing reaction was added to 5 µl of "stop solution" which consists of 3M sodium acetate [Sigma-Aldrich], 50 mM EDTA (Sigma-Aldrich) and glycogen (20 mg/ml) [Beckman] in a respective ratio of 2:2:1. To this, 60 µl of ice-cold 95% molecular grade ethanol (Sigma-Aldrich) was added and the samples were spun at 13,000 rpm at 4°C for 30 min to precipitate the DNA. The DNA pellet was washed twice with 200 µl of ice-cold 70% molecular grade ethanol (Sigma-Aldrich), spun at 13,000 rpm at 4°C for 5 min and then vacuum dried

for 15 min. DNA pellets were resuspended in 35 µl of deionised formamide (Beckman) and loaded onto the automated sequencer. The Beckman CEQ2000 uses a capillary system to electrophorese the sample through a polyacrylamide gel contained inside the capillary. In the sequencing reaction mix, chain terminating nucleotide bases labelled with different fluorescent tags are present and are incorporated into the DNA during PCR amplification. During electrophoresis a laser reads these fluorescent bases to determine the sequence of the sample.

Raw chromatograph data were analysed using 'SeqMan' sequence analysis software and multiple Clustal V alignments were made in the 'MegAlign'program, both programs were from the LASARGENE sequence analysis package (DNAstar Inc., Madison, WI, USA). Predicted aa sequences for the gene sequences in each ORF (*S* and *Pol*) were generated in the EMBOSS suite of programs held at the HPA Bioinformatics unit website (http://biosoftware.phls.org.uk) using the program PlotORF which allows aa translations to be made for all theoretically possible reading frames.

2.1.8: Culture of Eukaryotic cell lines

Upon receipt of a cell line, media were immediately tested for the presence of mycoplasma contamination by the use of the VenorGeM® Mycoplasma PCR Detection Kit (Sigma-Aldrich) following the manufacturers guidelines. American Type Culture Collection (ATCC)-recommended conditions were consulted for all cell lines. Cos-7, HepG2, Huh-7, 293T and CHO cell lines were all maintained in Dulbecco's Minimum Essential Medium (DMEM) [Gibco BRL] supplemented with 4500 mg/ml glucose, L-glutamine, sodium pyruvate and 10% FCS (heat inactivated) [Gibco BRL] overnight at 37°C with 5% CO_2 with no addition of antibiotics nor antifungals. Cells were passaged every 3-4 days when they reached 75% confluence. Medium was discarded and the cells

washed twice with 2 ml PBS (Gibco BRL). 2 ml 5% trypsin (Gibco BRL) in Versene (Gibco BRL) was added to the monolayers which were then agitated by tapping the flask until they sloughed off. Trypsinised cells were placed in fresh medium and cell clumps were separated into single cells by passing them repeatedly through a 10-ml pipette. Cells were then re-seeded into new flasks or plates at the desired density and incubated thereafter. Medium was replaced as necessary, typically every 2-3 days.

For liquid nitrogen storage, cells were re-seeded to a density of 3 x 10^4/ml in 'freezing medium', which consisted of 10% DMSO and 90% foetal calf serum [FCS] (heat inactivated). 1 ml vials of cells in freezing medium were placed in a Styrofoam box overnight at -80°C and then placed in liquid nitrogen storage in the vapour phase. Cells stored in liquid nitrogen were reactivated by immediately thawing at 37°C and were then pelleted by placing in a centrifuge at 37°C at 800 rpm for 5 min thus allowing freezing media containing DMSO to be decanted and the cells cultured in standard media. Cells reactivated from liquid nitrogen storage were always tested for mycoplamsa contamination upon revival. Cell viability was assayed by staining with 0.4% trypan blue (Gibco, BRL), which was mixed in a 1:1 ratio with the media to be tested. This was then mixed by tapping and left for 3 min to allow staining to occur. The cell mixture was then placed in a haemocytometer viewed under a light microscope, allowing the proportion of viable cells (stained blue) to non-viable cells (unstained) to be calculated.

2.1.9: Transfection of cells using lipofectamine LF2000 (Invitrogen)

Cells were seeded at a concentration of 5 x 10^4 viable cells/ml into 6 well plates in 2 ml growth media (DMEM/10% FCS [section 2.1.8]) with no antibiotics (if lipofection is carried out in the presence of antibiotics there is a potential for cell toxicity). Cells were

cultured to a density of 90% confluence (typically this occurred 1 d after seeding). 50 µl Optimem (Invitrogen) serum-free medium was mixed with 3 µl LF2000 lipofectamine transfection reagent (Invitrogen) per well and incubated at room temperature for 5 min. 1 µg pBK-CMV[smallS] DNA per well was added to the LF2000/Optimem mixture and incubated for 20 min. The DNA/LF2000/Optimem mixture was then added to the cells. Transfection controls consisted of supernatants derived from cultures which underwent a 'mock transfection', i.e., cells transfected with the 'empty' vector DNA with no insert. Additional controls included: cells transfected with the transfection reagent but no vector DNA; cells transfected with vector DNA but no transfection reagent; and cells which had undergone no transfection at all but which had been cultured alongside transfected cells. Cells were then grown for 2 d under standard culture conditions. The post-transfection supernatant was then collected, clarified by centrifugation for 5 min at 800 rpm and stored at -20°C. Cell pellets were collected by washing the residual monolayer four times in 500 µl PBS to prevent contamination from supernatant derived HBsAg whereupon cells were trypsinised using 500 µl 5 % trypsin (Gibco BRL) in Versene (Gibco BRL). Cells that sloughed off were harvested and added to 1 ml of PBS centrifuged for 5 min at 10,000 rpm, after which the supernatant was decanted, leaving cell pellets, which were then stored at -20°C.

2.1.10: Murex Ge34/36 HBsAg ELISA

The presence of HBsAg in supernatant or lysates of transfected cells or in HBsAg standards was assayed with the Murex Ge34/36 HBsAg ELISA. Ge34/36 is a sandwich assay that uses three monoclonal antibodies (MAbs): D2H5 (Tedder *et al.*, 1983); H3F5 (Tedder *et al.*, 1983); and P2D3 (Ijaz *et al.*, 2003). The MAbs recognise HBsAg and are bound to the solid phase at concentrations of 1 µg/ml for P2D3 and H3F5, and at 0.2 µg/ml for D2H5. (Figure 2.1). Purified goat anti-HBs conjugated to horseradish

peroxidase is used for the detection of capture antibody-antigen complexes detection, so HBsAg present in the sample is eventually held in an antibody-antigen-antibody-enzyme complex.

100 µl of the antigen preparation was initially bound to the MAbs on the solid phase by incubation of the sample at 37°C for 1 h. 100 µl of the polyclonal anti-HBs conjugate was then added to the sample in the well for further incubation at 37°C for 1 h. Following incubation of the antigen and conjugate the wells were washed 5 times (1 min each) with glycine/borate wash buffer (10 mM glycine, 10 mM Tris-borate, pH 8.0). 50 µl of 3, 3' 5, 5' tetramethylbenzidine (TMB) and hydrogen peroxide was added; wells containing the enzyme-conjugate complex bound to captured HBsAg turn blue/green in colour. 50 µl of 1 M sulphuric acid was then added to stop the reaction (indicated by a colour change to orange). The density of the orange colour was measured spectrophotometrically at 450 nm absorbance with a 620-nm reference wavelength, thereby allowing the detection of HBsAg in the well. All samples were assayed twice in duplicate wells to ensure reproducibility. Positive and negative controls provided with the kit were used in conjunction with positive controls using a quantified patient-derived HBsAg standard 'DA' at varying concentrations (section 2.5.1) and a negative control consisting of DMEM/FCS (10%). When samples derived from the transfection of cell cultures were assayed, the appropriate transfection controls were also assayed (section 2.1.9 and 2.5.8)

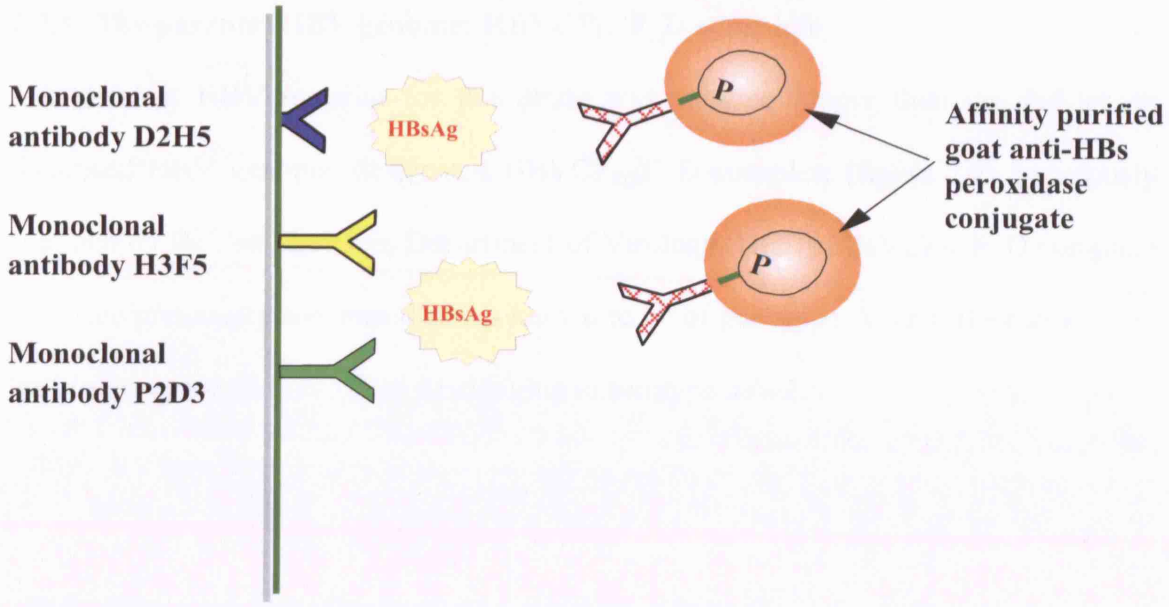


Figure 2.1 – Configuration of Murex Ge34/36 HBsAg ELISA

2.2: Construction of transfection vector bearing HBV small surface gene

2.2.1: The parental HBV genome: HBVCP$_{IY}$/F_D complete

The parental HBV material for this study was a twice longer than the full-length linearised HBV genome, designated HBVCP$_{IY}$/F_D complete (figure 2.2) [graciously supplied by Dr Tim Harrison, Department of Virology, UCL]. HBVCP$_{IY}$/F_D complete had been previously sequenced and is known to be of genotype A, and, if expressed, to lead to the production of HBsAg belonging to serotype *adw2*.

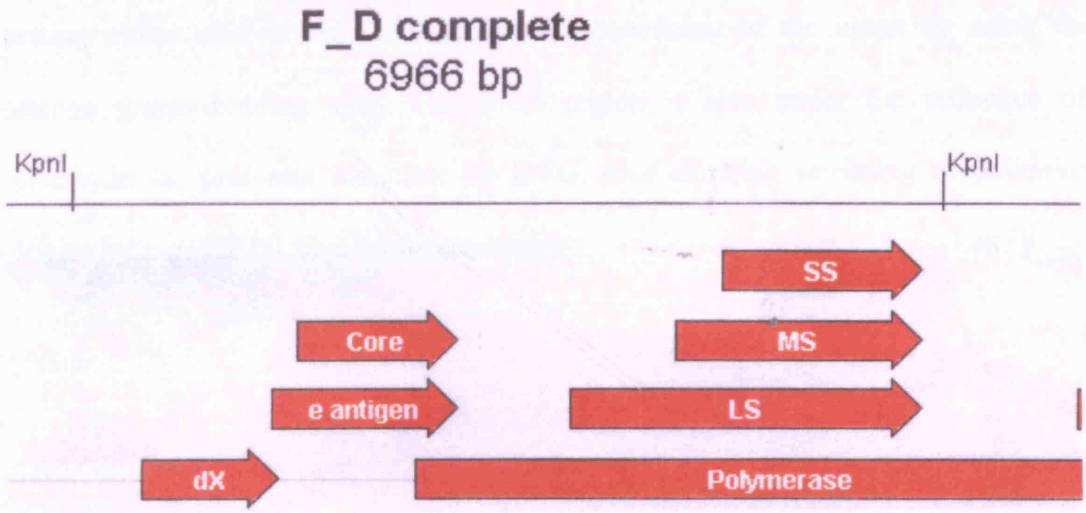
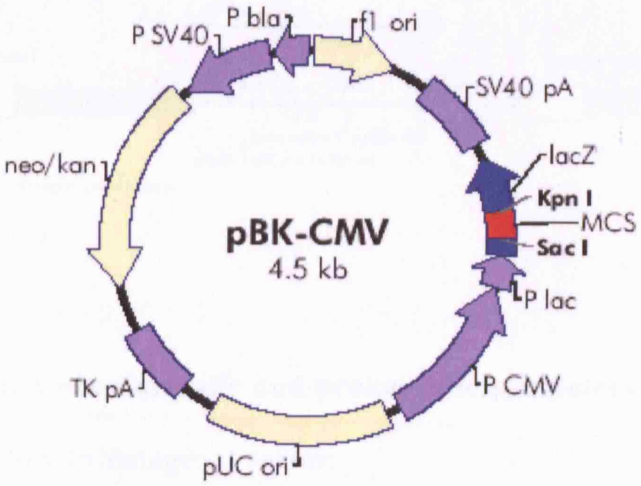


Figure 2.2 - Genomic arrangement of HBVCP$_{IY}$/F_D, the complete parental genome

2.2.2: Vector pBK-CMV

The phagemid transfection vector pBK-CMV (Stratagene) was chosen for delivery of cloned HBV. pBK-CMV (figure 2.3) is derived from a high copy-number pUC-based plasmid that allows convenient maintenance in prokaryotic cells. The presence of kanamycin and neomycin resistance genes also allows selection of transfected cells in both prokaryotic and eukaryotic cells. G418 is an analogue of neomycin sulphate that interferes with the function of 80S ribosomes and protein synthesis in eukaryotes; hence the presence of a neomycin resistance gene confers resistance and enables consequent selection of stable transfected eukaryotic cells. pBK-CMV contains a multiple cloning site (MCS) [figures 2.3 and 2.4], a region with many common restriction sites that affords ease of cloning into a region under the CMV IE promoter and so allows cloned gene expression in eukaryotic cells. The presence of M13 T3 and T7 promoter sites flanking either side of the MCS facilitates sequencing of the insert by using these common primer-binding sites. The MCS region is also under the influence of a prokaryotic *lac* promoter inducible by IPTG, thus allowing screening of prokaryotic colonies by x-gal/IPTG blue/white screening.



f1 origin 24–330
SV40 polyA 469–750
β-galactosidase α-fragment 812–1183
multiple cloning site 1015–1122
***lac* promoter** 1184–1305
CMV promoter 1306–1895
pUC origin 1954–2621
HSV-TK polyA 2760–3031
neomycin/kanamycin resistance ORF 3209–4000
SV40 promoter 4035–4373
***bla* promoter** 4392–4518

pBK-CMV Multiple Cloning Site Region
(sequence shown 952–1196)

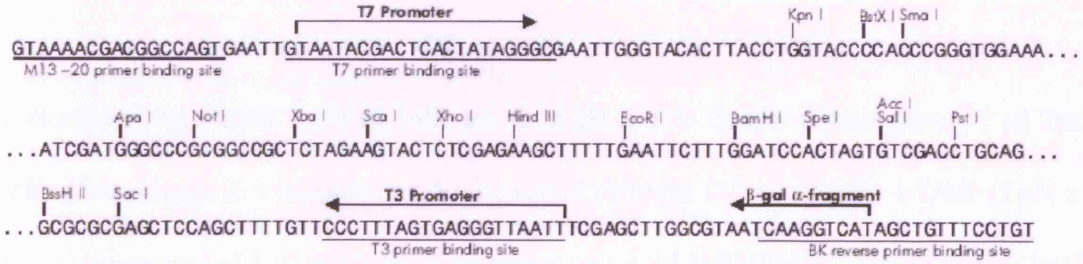


Figure 2.3 – Vector map and multiple cloning site (MCS) of pBK-CMV (Stratagene).

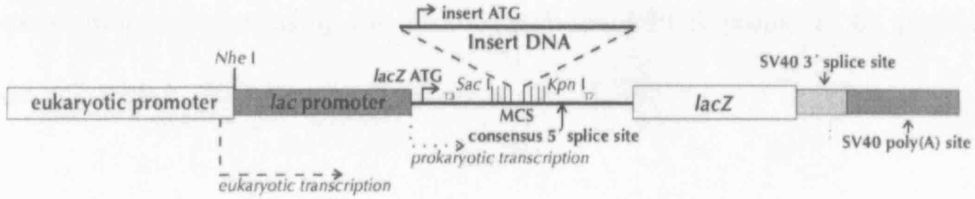


Figure 2.4 – Location of eukaryotic and prokaryotic promoters in relation to the MCS of the pBK-CMV (Stratagene) vector.

2.2.3: PCR Amplification of HBV small surface gene from parental genome

The oligonucleotide primers used for PCR amplification of the small S gene from $HBVCP_{IY}$ F_D (synthesised by Cruachem) are as follows:

Sense

5' GGGTCCCATATTTCTTGGGTACC 3'

Antisense

5' GTGAAAAGGGGGCAGCAAAGC 3'

The reaction conditions were as follows: 5 µl 10 X *Pfu* Buffer (Stratagene), 5 µl 2mM dNTPs (Promega), 2.5 µl sense and anti-sense primers (10 pmol/µl), 1 Unit (1µl) *Pfu* Turbo (Stratagene), 33 µl nuclease-free water and 1 µl $HBVPV_{IY}F\_D$ complete ($1x10^{-4}$ µg/ml) resulting in a final reaction volume of 50 µl. The thermocyling conditions for the reaction were: 94°C for 5 m, then 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min followed by a final extension of 72°C for 5 min. The PCR amplicon was purified

from reaction solution using the Promega 'Wizard PCR preps' DNA purification kit (section 2.1.3).

2.2.4: Vector quality assay

Vector quality was assessed by performing a single *Kpn1* digest. The reaction conditions were as follows: 1 µg pBK-CMV (1 µg/µl) was added to 1 µl *Kpn1* (New England Biolabs), 1 µl NE1 buffer, 1 µl BSA (1 mg/ml), 1 µl RNAase A and 5 µl nuclease-free water, giving a total reaction volume of 10 µl. The reaction was incubated for 2 h at 37°C. *Kpn1*-treated and untreated pBK-CMV was then subjected to agarose gel electrophoresis (section 2.1.2).

2.2.5: *Kpn1* and *Xho1* digest of pBK-CMV

In order to introduce complementary cloning sites vector pBK-CMV was digested by restriction enzymes *Kpn1* and *Xho1* as follows: 1 µg pBK-CMV (1 µg/µl) was added to 0.5 µl *Kpn1* and 0.5 µl *Xho1* (New England Biolabs), 1 µl NE2 buffer, 1 µl BSA (1 mg/ml), 1 µl RNAase A and 4 µl nuclease-free water, giving a total reaction volume of 10 µl. The reaction was incubated for 2 h at 37°C. *Kpn1* and *Xho1* -treated and untreated pBK-CMV was subjected to LMP agarose gel electrophoresis to confirm successful digest (section 2.1.2). Successfully cut vector was then excised from the gel using a sterile scalpel and purified using the Promega 'Wizard Preps' kit (section 2.1.3).

2.2.6: Dephosphorylation of *Kpn1* and *Xho1* -digested vector

Dephosphorylation of *Kpn1* and *Xho1* -cut, purified pBK-CMV was performed using shrimp alkaline phosphatase (SAP) as follows: 50 µl DNA (eluted in nuclease-free water) from the Wizard Preps purification procedure (section 2.1.3) was mixed with 5.5

µl 10 X SAP buffer, 5.5 µl SAP (New England Biolabs) to a total volume of 61 µl. The reaction mixture was then incubated for 2 h at 37°C followed by 15 min at 65°C.

2.2.7: *Kpn1* and *Xho1* digestion of HBV PCR amplicons

To introduce restriction sites complementary to those on the *Kpn1* and *Xho1* -cut vector, the small S gene PCR amplificon was also cut with *Kpn1* and *Xho1* under the following conditions: 21 µl DNA (*ca.* 100 µg/µl) was mixed with 3 µl *Kpn1*, 3 µl buffer NE2 and 3 µl BSA to a total reaction volume of 30 µl, which was then incubated for 2 h at 37°C.

2.2.8: Ligation of vector and amplicon digest products

The *Kpn1* and *Xho1* -cut vector and the amplicon were then ligated as follows: 1 µl *Kpn1* and *Xho1* -cut, dephosphorylated, purified vector (120 µg/µl) was mixed with 7 µl (80 µg/µl) of *Kpn1* and *Xho1* -cut purified amplicon, 1 µl T4 DNA ligase (New England Biolabs) and 1 µl T4 Buffer, giving a total volume of 10 µl for the whole reaction which was then incubated at 16°C overnight (c. 16 h). As control, the same reaction was also performed substituting 7 µl nuclease-free water. The ligation reaction product was then used to transform One Shot™ INVαF'/Top10 chemically competent cells (section 2.3). As controls, cells were also transfected with: *Kpn1* and *Xho1* -cut, dephosphorylated, purified vector without the amplicon: *Kpn1* and *Xho1* -cut purified amplicon with no vector; and nuclease-free water. Colonies were then grown in overnight culture and underwent Qiagen miniprep plasmid extraction (section 2.1.5).

2.2.9: Confirmation of ligation

Plasmids derived from the ligation reaction were then subjected to electrophoresis (section 2.1.2) to confirm mass and hence ligation. Ligation success was also assayed by performing *Kpn1* and *Xho1* digestions to confirm the presence of the cassette within

the vector. Ligation reaction-derived plasmids were subjected to the following digestion reaction: to 1 μg plasmid (1 μg/μl) was added 0.5 μl *Kpn1* and 0.5 μl *Xho1*, 1 μl NE2 buffer, 1 μl BSA (1 mg/ml), 1 μl RNAase A and 4 μl nuclease-free water, giving a total reaction volume of 10 μl. The reaction was incubated for 2 h at 37°C. The product was then assayed by agarose gel electrophoresis (section 2.1.2).

2.2.10: Confirmation of HBV cassette orientation

Confirmation of the small S gene cassette orientation within the vector was obtained by performing a *BamH1* and *Xcm1* -restriction digest. Plasmid derived from the ligation reaction was thus subjected to the following reaction: 3 μl DNA (0.33 μg/μl) was added to 1 μl *BamH1* (New England Biolabs), 1 μl *Xcm1* (New England Biolabs), 1 μl buffer NE2, 1 μl BSA and 3 μl nuclease-free water. The reaction was then incubated for 2 h at 37°C and the product then assayed by agarose gel electrophoresis (section 2.1.2).

2.2.11: DNA sequencing of HBV cassette

The nucleotide sequence of the HBV small surface gene cassette within vector pBK-CMV was confirmed by DNA sequencing (section 2.1.7) using the T3 and T7 promoter sites as sequencing primer-binding sites (figure 2.3). The sequences of these primers are as follows:

T3 promoter primer sequence:

5'-ATTAACCCTCACTAAAGGGA-3'

*T7 promoter primer sequence**:

5'-TAATACGACTCACTATAGGG-3'

2.2.12: Phylogenetic analysis of HBV cassette

The HBV cassette sequence was analysed within the Lasergene DNAstar* suite of programmes (section 2.1.7). Care was taken to remove all vector sequence from the sequence before analysis. The sequence was aligned against the small S gene from $HBVCP_{IY}$/F_D complete as well as GenBank sequences from prototypic genotypes (A-H); Genotype A, GenBank Accession AJ309371; Genotype B, GenBank Accession HPBADW1; Genotype C, GenBank Accession AY040627; Genotype D, GenBank Accession AB033558; Genotype E, GenBank Accession HHVBE4; Genotype F, GenBank Accession HBVFFOU; and Genotype G, GenBank Accession AP007264; and Genotype H, GenBank Accession HY090459. A distance matrix of the cassette sequence and the GenBank type sequences in alignment was calculated and used to draw a neighbour joining tree with MegAlign program (Lasergene). Small S gene DNA that matched the sequence of $HBVCP_{IY}$/F_D complete and cloned into pBK-CMV in the correct orientation was designated pBK-CMV[smallS].

2.2.13: Predicted serotype of HBV cassette

The predicted serotype of the HBV small S gene based on nucleotide sequence was determined according to Norder *et al* (1992 and 1993) (figure 2.5).

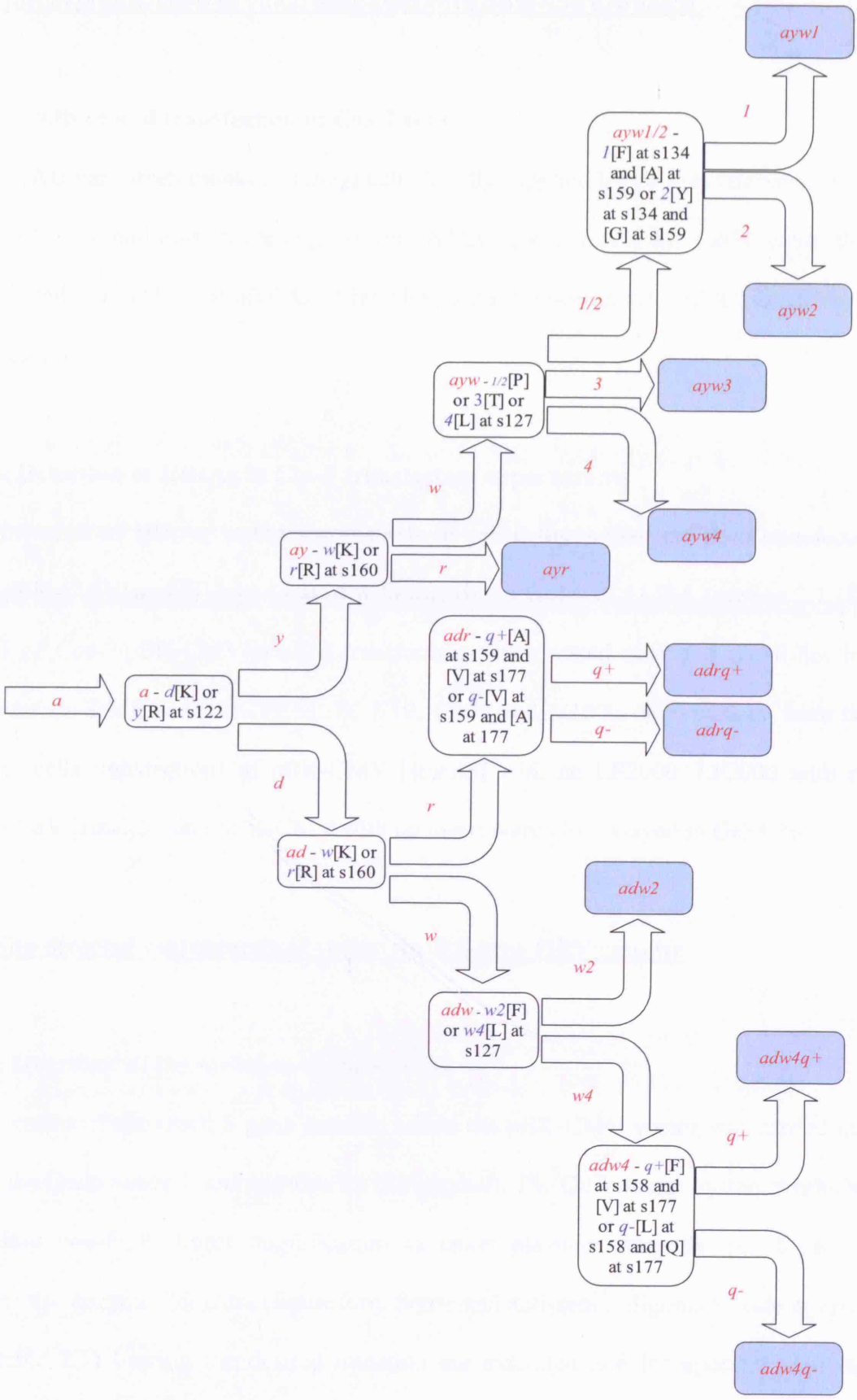


Figure 2.5 – Algorithm to determine serotype from the S gene sequence

2.3: Initial transfection of eukaryotic cells with pBK-CMV[smallS]

2.3.1: Culture and transfection of Cos-7 cells

Cos-7 (African Green monkey kidney) cells (kindly supplied by Dr Tim Harrison,) were cultured in conditions according to the ATCC (section 2.1.8). Cells were then transfected with wild type pBK-CMV[smallS] using lipofectamine LF2000 (Invitrogen) [section 2.1.9].

2.3.2: Detection of HBsAg in Cos-7 transfectant supernatants

The presence of HBsAg in the supernatants of cell cultures that had been transfected with pBK-CMV[smallS] was assayed with the Murex Ge34/36 ELISA (section 2.1.10). 50 µl of Cos-7/pBK-CMV[smallS] transfectants were tested neat and the following dilutions (in DMEM + 10% FCS): ½, 1/10, 1/100 and 1/1000. Supernatants from the control cells transfections of pBK-CMV [smallS] with no LF2000, LF2000 with no pBK-CMV [smallS], and pBK-CMV with no insert were also assayed in Ge34/36.

2.4: Site-directed mutagenesis of vector small S gene HBV cassette

2.4.1: Overview of the mutagenesis procedure

Mutagenesis of the small S gene cassette within the pBK-CMV vector was carried out using the Quikchange II mutagenesis kit (Stratagene). The Quikchange system works by an initial non-PCR, linear amplification of target plasmid using the proof-reading polymerase enzyme *Pfu* Ultra (figure 2.6). Sense and anti-sense oligonucleotide primers (see table 2.1) bearing the desired mutation are extended and incorporated into the nascent plasmid DNA stands. The polymerase reaction mixture is then subjected to digestion with the restriction enzyme *Dpn1*; this being a methylation dependant

enzyme, the dam-methylated parental strands will be digested leaving the unmethylated strands generated from the mutagenic polymerase reaction. The remaining nicked circular DNA is then transfected into XL-1 Blue Supercompetent cells, which repair the nicks using bacterial ligases allowing replication of the plasmid to occur. Single bacterial colonies maintaining the plasmid can then be selected using an antibacterial resistance marker and the potentially mutated plasmid can then be extracted allowing a screening assay to determine if the clone carries the desired mutation. A list of the mutations to be engineered into the vector can be found in table 2.2.

Mutant	Antiviral associated with mutation	Reference
rtM204V/sI195M	lamivudine	Ling *et al.*, 1996; Tipples *et al.*, 1996
rtM204I/sW196S	lamivudine	Ling *et al.*, 1996
rtM204I/sW196L	lamivudine	Ling *et al.*, 1996
rtL180M/sSilent	lamivudine	Ling and Harrison, 1999
rtL180M/sSilent + rtM204V/sI195M	lamivudine	Ling and Harrison, 1999
rtV173L/sE164D	lamivudine	Ogata *et al.*, 1999; Delaney *et al.*, 2003
rtV173L/sE164D + rtM204V/sI195M	lamivudine	Delaney *et al.*, 2003
rtT128N/sP120T	lamivudine	Torresi *et al.*, 2002b
rtT128N/sP120T + rtM204V/sI195M	lamivudine	Torresi *et al.*, 2002b
rtSilent/sD144E	lamivudine	Torresi *et al.*, 2002b
rtSilent/sD144E + rtM204V/sI195M	lamivudine	Torresi *et al.*, 2002b
rtR153Q/sG145R	lamivudine	Torresi *et al.*, 2002b
rtR153Q/sG145R + rtM204V/sI195M	lamivudine	Torresi *et al.*, 2002b
rtR153K/sD144EsG145R	lamivudine	Torresi *et al.*, 2002b
rtR153K/sD144EsG145R + rtM204V/sI195M	lamivudine	Torresi *et al.*, 2002b
rtF166L/sF158Y	lamivudine	Terrault et al., 1998, Torresi et al., 2002a
rtF166L/sF158Y + rtM204V/sI195M	lamivudine	Torresi *et al.*, 2002a
rtA181T/sW172STOP	adefovir	Hadziyannis *et al.*, 2005
rtA181V/sS173F	adefovir	Hadziyannis *et al.*, 2005
rtI169T/sF161L	entecavir	Tenney *et al.*, 2004
rtT184S/sL176V	entecavir	Tenney *et al.*, 2004
rtS202I/sV194F	entecavir	Tenney *et al.*, 2004
rtS202I/sV194F + rtT184S/sL176V	entecavir	Tenney *et al.*, 2004
rtS202I/sV194F + rtT184S/sL176V + rtI169T/sF161L	entecavir	Tenney *et al.*, 2004

Table 2.1 – List of mutations to be engineered into pBK-CMV[smallS] via site- directed mutagenesis.

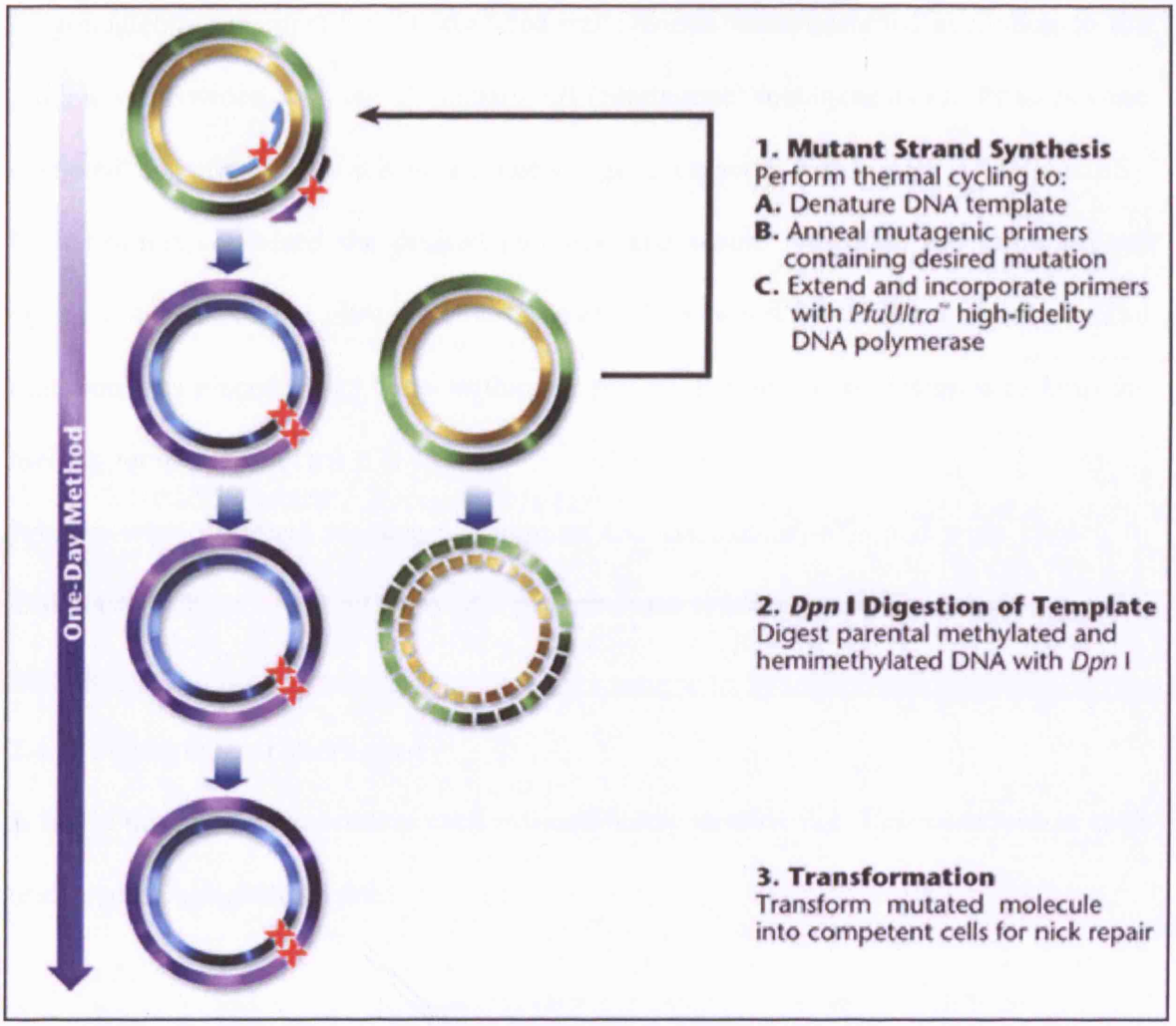


Figure 2.6 – Overview of the Quikchange II mutagenesis protocol (Stratagene)

2.4.2: Primer selection and design

Oligonucleotide primers for site-directed mutagenesis were designed according to the guidelines provided with the Quikchange II (Stratagene) mutagenesis kit. Primers were designed specifically for the small surface gene cassette within pBK-CMV[smallS]. Both primers contained the desired mutation and would anneal to the same site on opposite strands of the plasmid. Primers were all between 29-45 bases in length. The mutation was placed 10-15 bases within the primer. Primers were designed to keep the melting temperature (Tm) $\geq$ 78 °C.

Primers were designed to have a minimum GC content of 40% and were chosen to terminate in either a G or a C base. All primers were synthesised by Cruachem.

2.4.3: Mutagenic primers used

A list of the mutagenic primers used is found below in table 2.2. The mutations in each primer are highlighted in red.

Mutagenic Primer	**Nucleotide sequence (5' → 3')**
rtM204V/sI195M (sense)	TTTGGCTTTCAGTTATGTGGATGATGTGGTATTGGGGGCC
rtM204V/sI195M (antisense)	AATACCACATCATCCACATAACTGAAAGCCAAACAGTGGGG
rtM204I/sW196S (sense)	TGTTTGGCCTTTCAGTTATATCGATGATGTGGTATTGGGGG
rtM204I/sW196S (antisense)	TTGGCCCCCAATACCACATCATCGATATAACTGAAAGCC
rtM204I/sW196L (sense)	TGTTTGGCCTTTCAGTTATATTGATGATGTGGTATTGGGGG
rtM204I/sW196L (antisense)	TTGGCCCCCAATACCACATCATCATCAATATAACTGAAAGCC
rtL180M/sSilent (sense)	TGGGCCTCAGTCCGTTTCTCATGGCTCAGTTTACTAGC
rtL180M/sSilent (antisense)	TAAACTGAGCCAAGAGCCCCGGGCTGAGGCCC
rtV173L/sE164D (sense)	TTCGCAAAATTCCTATGGGATTGGGCCTCAGTCCG
rtV173L/sE164D (antisense)	ACGGACTGAGGCCCAATCCCATAGGAATTTTGCG

Table 2.2 – Oligonucleotide primers used in Quikchange II mutagenesis reactions.

(continued overleaf)

Mutagenic Primer	Nucleotide sequence (5' → 3')
rtT128N/sP120T (sense)	ACATCAACAAGCACGGGAACATGCAAGACCTGCAC AACTC
rtT128N/sP120T (antisense)	AGTTGTGCAGGTCTTGCATGTTCCCGTGCTTGTTGA
rtR153Q/sG145R (sense)	TTGCTGTACAAAACCTTCGGACAGAAACTGCACCT GTATTCCC
rtR153Q/sG145R (antisense)	TGGGATGGGAATACAGGTGCAGTTTCTGTCCGAAG GTTTTGTCAG
rtR153K/sD144E+sG145R (sense)	TGCTGTACAAAACCTTCGGAAAGAAACTGCACCTG TATTCCC
rtR153K/sD144E+sG145R (sense)	TACAGGTGCAGTTTCTTTCCGAAGGTTTTTGTACAG CAACAAG
rtSilent/sD144E (sense)	TTGCTGTACAAAACCTTCGGAAGGAAACTGCACCT GTATTCCC
rtSilent/sD144E (antisense)	AATACAGGTGGATTTCCTTCCGAAGGTTTTGTACAG CAAC
rtF166L/sF158Y (sense)	TCCCATCATCCTGGGCTTACGCAAAATTCCTATG
rtF166L/sF158Y (antisense)	TCCCATAGGTTTTGCGTAAGCCCAGGATGATG

Table 2.2 (cont). – Oligonucleotide primers used in Quikchange II mutagenesis reactions. (continued overleaf)

Mutagenic Primer	Nucleotide sequence 5' → 3'
rtM204V/sW196STOP (sense)	TGTTTGGCCTTTCAGTTATATAGATGATGTGGTATTGGGGG
rtM204V/sW196STOP (antisense)	TTGGCCCCCAATACCACATCATCTATATAACTGAAAGCC
rtA181V/sL173F (sense)	TCCGTTTCTCCTGGTTCAGTTTACTAGCGC
rtLA181V/sL173F (antisense)	TAGTAAAGTGAACCAGGAGAAACGGGAC
rtA181T/sW172STOP (sense)	TCCGTTTCTCCTGACTCAGTTTACTAGCGC
rtA181T/sW172STOP (antisense)	TAGTAAAGTGAGTCAGGAGAAACGGGAC
rtS202I/sV194F (sense)	ACTGTTTGGCTTTCATTTATATGGATGATGTGGTATTGG
rtS202I/sV194F (antisense)	AATACCACATCATCCATATAAATGAAAGCCAAACAGTGG
rtT184S/sL176V (sense)	TTTCTCCTGGCTCAGTTTAGTAGCGCCAGTGG
rtT184S/sL176V (antisense)	AACAAATGGCGCTACTAAACTGAGCCAGGAGAAACGG

Table 2.2 (cont.) – Oligonucleotide primers used in Quikchange II mutagenesis reactions. (continued overleaf)

Mutagenic Primer	Nucleotide sequence (5' → 3')
rtI169T/sF161L (sense)	TCATCCTGGGCTTTCGCAAAACTCCATGGGAGTGGGCC
rtI169T/sF161L (antisense)	TGAGGCCCACTCCCATAGGAGTTTTGCGAAAG

Table 2.2 (cont). – Oligonucleotide primers used in Quikchange II mutagenesis reactions.

2.4.4: Mutagenesis reaction conditions

These are as follows. The mutagenic reaction mix contained 1 µl pBK-CMV[smallS] (c. 30 ng /µl), 2.5 Unit (1 µl) *Pfu* Ultra, 1 µl dNTPs (20 mM), 1 µl each mutagenic primer (100 ng /µl), 5 µl Reaction Buffer 10 X (Stratagene) (which contains $MgCl_2$). The cycling conditions were: an initial denaturation of 95°C for 30 s followed by 16 cycles of 95°C for 30 s, 55°C for 1 min and 68°C for 1 min and concluded with a hold of 4°C. Following the mutagenic reaction, 1 µl (10 U) of *Dpn1* was added to the reaction mixture, which was then centrifuged in a tabletop Pico centrifuge at 5000 rpm for 1 min then incubated at 37°C for 1 h followed by 4°C for 10 min. XL-1 Blue Supercompetent cells were transformed by the following process; cells were thawed on ice, which were then aliquoted in 50-µl batches into Falcon® 2059 polypropylene tubes. Cells were then mixed with 1 µl *Dpn1*-treated reaction mixture and incubated for 30 min on ice. The tube was then heat-pulsed for 45 s at 42°C thereafter quenched in ice for 2 min. 0.5 ml of NZY^+ broth that had been preheated to 37°C was added to the tube that was incubated horizontally for 1 h at 37°C with 225 rpm shaking. The entire transformation reaction was then plated onto a LB Agar plate containing kanamycin (50 µg/ml) and

incubated overnight (c.16 h) at 37°C. Four colonies per plate were picked and grown in overnight culture in 5 ml LB Broth containing kanamycin (50 μg/ml) at 37°C shaken at 225 rpm. 3 ml of the overnight culture was centrifuged at 13,000 rpm for 5 min to pellet the cells and the supernatant discarded. The plate was stored at 4°C for retrieval of colonies harbouring correctly mutated plasmid. Plasmids were isolated from the cells using the Qiagen Miniprep Plasmid isolation protocol (section 2.1.5). Potentially mutated plasmids were screened for the presence of the mutation by DNA sequencing using primers T7 and T3 (section 2.1.7, figure 2.3)

2.4.5: Mutant bacterial stocks

Once a mutant plasmid sequence had been confirmed, bacterial glycerol stocks and plasmid stocks were prepared from the initial colony stored on the LB-Kanamycin plate. The colony was picked using a sterile pipette tip and placed in overnight culture in 5 ml LB Broth with kanamycin (50 μg/ml); from this, 500 μl of culture was mixed with an equal volume of glycerol, mixed, placed in a polystyrene boxed and stored at 80°C. The remaining 4.5 ml of the overnight culture was used to extract highly purified plasmid suitable for eukaryotic cell transfection using the Qiagen 'Endo Free' maxiprep kit (section 2.1.6)

2.4.6: Multiple mutations of the same vector

Double mutations were created by performing the mutagenesis reaction for the first desired mutation. Once completed this first mutant was then used as the parental strand in a second mutagenesis reaction using primers for the second desired mutation. Thus, the resultant plasmid would bear both mutations. As all the double mutations studied were more than 20 nucleotides apart, it was not necessary to construct specific primers for any double mutants.

2.5: Optimisation of transfection of eukaryotic cells

2.5.1: Preparation of a standard curve of HBsAg concentration versus absorbance with the Ge34/36 ELISA

A standard curve of Ge34/36 ELISA absorbance for varying concentrations of wild type HBsAg was prepared through the use of the DA HBsAg serum standard. The DA HBsAg standard had previously been quantified against WHO HBsAg standards, and HBsAg concentration in the sample had been calculated as 2.56 x 10^5 ng/ml HBsAg. From the DA standard, dilutions in DMEM/FCS (10%) were made to achieve 10, 5, 3, 2, 1 and 0.5 ng/ml HBsAg. The dilution series was prepared on 3 separate occasions, and the series from each of these occasions were assayed 4 times in the Ge34/36 ELISA (section 2.1.10). The standard curve was plotted from the mean results of the dilution series.

2.5.2: Culture of HepG2 cells

HepG2 cells [of HCC origin] (Knowles *et al.*, 1980) {provided by Dr Sam Coward, Department of Hepatology Royal Free and University College Medical School} were tested for the presence of mycoplamsa and cultured according to ATCC guidelines for the cell line (section 2.1.8).

2.5.3: Culture of 293T cells

293T cells [of human kidney origin] (Xie *et al.*, 1996) {provided by Arinder Kohli, Wohl Virion Institute, UCL} were tested for the presence of mycoplasma and then cultured according to ATCC guidelines for the cell line (section 2.1.8).

2.5.4: Culture of Cos-7 cells

Cos-7 cells [of African green monkey kidney origin] (Gluzman, 1981) {provided by Dr Tim Harrison, Department of Virology, UCL} were tested for the presence of mycoplamsa and then cultured according to ATCC guidelines for the cell line (section 2.1.8).

2.5.5: Culture Huh-7 cells

Huh-7 cells [of HCC origin] (Cheng *et al.*, 1993) {provided by Dr Nikolai Nauomov, Institute of Hepatology, UCL} were tested for the presence of mycoplasma and then cultured according to ATCC guidelines for the cell line (section 2.1.8).

2.5.6: Culture of CHO cells

CHO cells [of Chinese Hamster Ovary origin] (Puck *et al.*, 1958) {provided by Dr Bridgette Ferns, Department of Virology, UCL} were tested for the presence of mycoplamsa and then cultured according to ATCC guidelines for the cell line (section 2.1.8).

2.5.7: Transfection of HepG2 cells with Lipofectamine LF2000

HepG2 cells were transfected with pBK-CMV[smallS] (section 2.1.9), with concentrations of 1 μg pBK-CMV[smallS] per well or 4 μg pBK-CMV[smallS] per well. Both transfection concentrations used a 3:1 ratio of LF2000 per μg of pBK-CMV[smallS] DNA.

Supernatants were then assayed at neat or a 1/10 dilution (in DMEM/FCS) in the Ge34/36 ELISA (section 2.1.10) and the absorbances measured on the Ge34/36 standard curve (section 2.5.1) to calculate the amount of HBsAg shed.

2.5.8: Transfection of cells with FuGene 6 (Roche) transfection reagent

Cells were seeded at a concentration of 5 X 10^4 viable cells/ml into 6 well plates in 2 ml growth media (DMEM/10% FCS [section 2.1.8]) with no antibiotics (transfection carried out in the presence of antibiotics may exert cell toxicity). Cells were cultured to a density of 50-80% confluence (typically this occurred 1 d after seeding). The required amount of FuGene 6 for each transfection was added to Optimem serum-free medium (Gibco BRL) to a final volume of 100 µl per well (of a 6-well plate) taking care that the pipette tip did not touch the wall of the tube. This was mixed by flicking and incubated at room temperature for 5 min. 1-4 µg plasmid DNA per well was then added to FuGene6 in ratios of 6:1, 3:1, 2:1 3:2 or 1:1 of Fugene 6 µl to plasmid DNA µg. This was again mixed by flicking and incubated for 30 min at room temperature. The mixture was then gently added to the cell wells. Transfection controls included consisted of supernatants derived from cultures which underwent a 'mock transfection', with: cells transfected with the pBK-CMV vector without insert; cells transfected with the transfection reagent but no pBK-CMV[smallS] DNA; cells transfected with pBK-CMV[smallS] DNA but with no transfection reagent; and cells which had undergone no transfection at all but which had been cultured alongside transfected cells. Cells were then grown for the desired number of days (2 or 6) under standard culture conditions. Every other day 200 µl DMEM/FCS (10%) was added to the each well to maintain media volume due to loss from evaporation. When cells were grown in extended time course testing, 100 µl of supernatants was removed daily for future ELISA analysis the same volume (100 µl) of DMEM/FCS (10%) was added to replace the removed media. Post-transfection supernatants were collected, clarified by centrifugation for 5 min at 800 rpm and stored at -20°C. Cell pellets were collected by washing the residual monolayer 2 X in 500 µl PBS followed by trypsinisation using 500 µl 5 %

typsin/versene. Cells that sloughed off were harvested, added to 1 ml of PBS centrifuged for 5 min at 10,000 rpm and supernatant was decanted leaving cell pellets, which were stored at -20°C. Supernatants were then assayed at neat or a 1/10 dilution (in DMEM/FCS) in the Ge34/36 ELISA (section 2.1.10) and the absorbances measured on the Ge34/36 standard curve (section 2.5.1) to determine the amount of HBsAg shed.

2.5.9: Non-denaturing lysis of cell pellets

The Cell Lytic MT mammalian cell lysis kit (Sigma Aldrich) was used for the lysis of transfected cells. Lysis buffer was prepared by adding 200 µl Tris-EDTA, 200 µl 150 mM NaCl, 100 µl 1% IPEGAL (0.1% final concentration), 100 µl 1% Tween 20 (0.1% final concentration), 10 µl protease inhibition mix (consisting of 4-(2-aminoethyl) benzenesulphonate [AEBSF], pepstatin A, E-64, bestatin, leupeptin and aprotinin) and 400 µl distilled water giving a total of 1 ml lysis buffer per lysis reaction. The lysis buffer was stored at 4 °C.

Tween 20 and IPEGAL were chosen for non-denaturing lysis as they are both less active non-ionic detergents, so their use at a low final concentration (0.5%) would allow cell outer membrane lysis to occur, thereby releasing any internal HBsAg, but ensuring no significant disruption to HBsAg associated with internal membranes. EDTA was used in the lysis buffer to chelate Ca^{+} ions involved in intercellular and intra-cellular adhesion and to improve cellular break down, it would also chelate ions which were co-factors of certain enzymes thereby reducing their activity after lysis and possibly prevent degradation of HBsAg. Protease inhibitors were also used to prevent HBsAg being degraded. The different inhibitors used have different specificities for inhibiting certain families of proteases, e.g., PMSF for serine proteases. Moreover, control of adequate pH and osmolarity (Tris and NaCl) was needed to maintain conformation of

HBsAg so that it would not unfold and lose its antigenicity. The DTT reductant provided with the kit was not added as it would break cystine-to-cystine disulphide bonds essential for maintaining the native conformation of HBsAg.

Tubes containing cell pellets (previously stored at -20°C post-harvesting) were quenched in ice and cold lysis buffer was added. The tubes were briefly vortexed, and then mixed on ice for 30 min. The lysis mixture was then centrifuged at 13,000 rpm on a tabletop centrifuge at 4°C for 10 min. The supernatants were decanted to chilled tube and then assayed at neat concentration in the Ge34/36 ELISA (section 2.1.10). The remainder of the lysates were stored at -20°C.

2.5.10: Generation of stable transfected cells lines

HepG2, 293T, Cos-7, Huh-7 and CHO cell lines were cultured in standard conditions (section 2.1.8 and sections 2.5.2, 2.5.3, 2.5.4, 2.5.5 and 2.5.6, respectively) and seeded at a density of 5 x 10^4 into 24-well plates after which they were transfected with 4 µg pBK-CMV[smallS] using FuGene 6 (Roche) in a 6:1 reagent µl/DNA µg ratio (section 2.5.8). DMEM/FCS supplemented with G418 was added at a concentration of 800 µg/ml 1 day post transfection for selection. Cells were then maintained in culture with media replacement every day (to maintain G418 titres) until large amounts of cell death were observed (typically day 3 to day 7 post transfection though results varied for each cell line). Resistant colonies that remained could be identified in the culture vessel. These were then re-seeded at a concentration of 0.5 cell/100 µl in 100 µl per well in a 96-well plate and cultured in DMEM/FCS supplemented with G418 at a concentration of 300 µg/ml, then cultured in successively larger vessels until there were sufficient cells to freeze stocks (section 2.1.8). Cell culture supernatants were harvested and

clarified (section 2.1.9) and either stored at -20°C or their HBsAg content immediately measured in the Ge34/36 ELISA neat or as a 1/10 dilution (in DMEM/FCS),

2.5.11: Transfection of CHO cells with wild type and mutant plasmids

CHO cells were cultured under standard conditions (section 2.1.8) and transfected with 4 μg wild type and mutant (table 2.1) pBK-CMV[smallS] with 24 μl FuGene 6 (a 6:1 transfection reagent/DNA ratio) per well under conditions earlier specified (section 2.5.8). 8 wells were transfected per plasmid. Supernatants and lysates harvested (sections 2.1.9 and 2.5.9) 5 days post transfection were pooled with wells transfected with the same plasmid. The material was placed in aliquots and stored at -20°C. This entire transfection process was then repeated 3 times for each plasmid, with each transfection batch of pooled supernatants and cell lysates kept separate.

2.6: Optimisation of monoclonal and polyclonal HBsAg capture ELISAs

2.6.1: Increase in incubation times in Ge34/36 protocol

Using the DA HBsAg standard diluted to 3 ng/ml and 0.3 ng/ml HBsAg, Ge34/36 ELISA incubation times were assayed following 1 h incubation with sample at 37°C, and 1h conjugate incubation at 37°C followed by incubation with TMB for 30 min at 37°C. In order to increase sensitivity the standard Ge34/36 ELISA protocol was then compared to an extended incubation format in which the initial sample incubation time was increased to overnight at room temperature, and the conjugate incubation time was extended to 4 h. Hence the final protocol for the extended assay was an overnight (*ca.* 16 h) room temperature (*ca.* 20°C) sample incubation followed by a 4 h conjugate incubation at 37°C and a 30 min TMB incubation 37°C. Other than increases in

incubation times and a reduced incubation temperature for the initial sample incubation, nothing else was altered in the Ge34/36 ELISA (section 2.1.10).

2.6.2: Monoclonal antibody-capture ELISAs

The individual MAb capture ELISAs used MAb P2D3 (Ijaz *et al.*, 2003) which is known to bind to a linear epitope located on surface residues 121-128 (Ijaz *et al.*, 2003); MAb H3F5 (Tedder *et al.,* 1983) which is known to bind to an epitope on surface residues 131-142 (S. Ijaz, personal communication) and MAb D2H5 (Tedder *et al.,* 1983) which is known to bind surface residues 142-147 (S. Ijaz, personal communication). All MAbs were provided by Murex individually pre-coated onto 8 well strips (in a 96- well plate format) at concentrations of 1 µg/ml for P2D3 and H3F5, and at 0.2 µg/ml for D2H5. Samples were assayed on the individual MAb plates using the same reagents as the standard Ge34/36 ELISA (section 2.1.10) but with the use of extended incubation times (section 2.6.1). Pooled wild type, *in vitro*-generated HBsAg made by pBK-CMV[smallS] transfection of CHO cells (section 2.5.11) was initially assayed in the individual capture assay in a $1/10^{th}$ fold dilution series in 10% DMEM/FCS at concentrations from neat down to 1:9.

2.6.3: Coating ELISA solid phase with MAb D2H5

Purified MAb D2H5 was provided by Murex in solution in order that it may also be plated at a higher concentration than provided in the supplied pre-coated plates. The MAb was diluted to a concentration of 1 µg/ml in the following three ELISA plate coating buffers: 10 mM PBS, 10 mM Tris-HCL (pH 8.0) and carbonate buffer (0.1M Na_2CO_3 0.1 M $NaHCO_3$ pH 9.0). Sterilised 96-well plates (Sterilin) were blocked by incubation overnight (*ca.*16 h) at 4°C with 3% BSA in PBS. Plates were then washed 4 x 1 min in PBS. 100 µl of D2H5 at 1 µg/ml diluted in each of the 3 buffers was

incubated in the 96 well-plates at 37°C for 1 h. Plates were then washed 4 x 1 min in PBS. The DA HBsAg standard was then assayed at a starting concentration of 3 ng/ml in a one tenth fold dilution series in each of the three buffer plates using the standard Ge34/36 protocol (section 2.1.10). D2H5 was again diluted in the carbonate buffer at 1 µg/ml and incubated and blocked (see above) in 96 well plates at either 37°C for 1 h, room temperature (*ca.* 20°C) for 4 h or at 4°C overnight (*ca.* 16 h) and then washed 4 x 1 min in PBS. The DA HBsAg standard was then assayed at a concentration of 3 ng/ml in a one tenth fold dilution series in each of the 3 buffer plates using the standard Ge34/36 protocol (section 2.1.10).

2.6.4: Isolation of hyper-immune horse anti-HBs

Hyper-immune horse sera was prepeared from an animal that had been repeatedly immunised with HBV vaccine. Immunoglobulins (and therefore containing anti-HBs) were salted out from the serum by ammonium sulphate precipitation as follows. 500 µl serum was diluted 1 in 3 in 0.02 M Tris-HCL pH 8.0, then 2 ml saturated $(NH_4)_2SO_4$ was added drop-wise while vortexing to give a final concentration of 40%. This was spun for 10 min at 2000 rpm and the supernatant discarded. 3 ml 0.02 M Tris-HCL (pH 8.0) was added and the pellet resuspended; 2 ml $(NH_4)_2SO_4$ was again added drop-wise while vortexing and re-spun for 10 min at 200 rpm and the supernatant discarded. The pellet was resuspended in 1 ml of 10 mM PBS and dialysed overnight at 4°C in 1 L of 10 mM PBS.

2.6.5: Coating ELISA solid phase with hyper-immune horse anti-HBs

The hyper-immune horse immunoglobulin preparation was then diluted in carbonate buffer at dilutions of 1/500, 1/1000, 1/2000, 1/4000 and 1/16000, and used to coat

blocked 96 well plates (section 2.6.3) by incubation overnight at 4°C followed by a 4 X 1 min wash with PBS. Two dilutions of the DA HBsAg standard (section 2.5.1) were then assayed at concentrations of 3 ng/ml and 1 ng/ml following the extended overnight Ge34/36 incubation time protocol (section 2.6.1). Pooled cell culture supernatants from CHO cells transfected with wild type pBK-CMV[smallS] were also assayed in this manner for comparison (section 2.5.11).

2.6.6: ELISA Analysis of supernatants and lysates of cells transfected with mutant pBK-CMV[smallS] plasmids

The 3 supernatant pools obtained from cells transfected with mutant and wild type pBK-CMV[smallS] plasmids (section 2.5.11) were assayed from neat concentration across a fine gradient down to a $1/10^{th}$ dilution in the Ge34/36 ELISA using the extended incubation format (section 2.6.1). For testing against MAbs P2D3 and H3F5, supernatant pools were analysed using the plates pre-coated with antibody at 1 μg/ml provided by Murex assayed as $1/10^{th}$ fold dilutions using the Ge34/36 extended incubation protocol. Serial dilutions of mutant and wild type supernatant pools were assayed against D2H5 at a concentration of 1 μg/ml which had been coated in carbonate buffer overnight and also using the extended Ge34/36 protocol. The polyclonal assay was carried out in 96-well plates which had been coated with hyper-immune horse serum at a concentration of 1/500 following the extended Ge34/36 protocol. The 3 cell pellet lysates (section 2.5.3) pools from wild type and mutant plasmid transfected cells (section 2.5.11) were assayed at neat concentration in the Ge34/36 assay only, according to the extended incubation protocol.

2.6.7: Analyses of ELISA results from mutant samples

The results from the Ge34/36, MAb and polyclonal supernatant ELISAs, and the Ge34/36 cell pellet lysates ELISAs were analysed in Excel (Microsoft). Absorbance results from each of the three supernatant transfection pools (section 2.5.11) were used to calculate binding ratios obtained from each assay: Binding ratio here is defined as sample raw absorbance ÷ negative control absorbance (negative control = mock transfection with 'empty' pBK-CMV[smallS]). Standard deviation and mean binding ratio results were then calculated for each dilution point. The mean results were then plotted for each individual mutant for every ELISA, with the corresponding results for unmutated HBsAg included in every plot. A separate plot of each mutant against all ELISAs in one chart was also prepared. For ELISA of cell lysates, binding ratios were calculated using mock transfected cells as negative control. Binding rations for both lysates and supernatants were compared to the 'DA'patient derived HBsAg standard curve in order to deduce HBsAg concentration in ng/ml equivalents. Supernatant and lysate ELISA HBsAg concentrations were used to generate a 'supernatant/lysate ' HBsAg equivalent ratio in order to evaluate HBsAg secretion efficiency.

2.7: – Molecular Epidemiology of incident HBV infections

2.7.1: Selection of acutely infected patient serum samples

Acute infections were identified serologically on the basis of an anti-core IgM titre > 200 Paul Erlich institute International Units per millilitre (PEIU/ml). The Health Protection Agency (HPA) Sexually Transmitted and Blood-borne Virus (SBVL) Reference Laboratory database was accessed to identify patients who had been tested between 1997-2001 for serological markers of HBV infection and for whom sera were

still available in the serum archive. This process identified 427 patients with potentially acute HBV infection.

2.7.2: HBV serum standards

A quantified HBV serum DNA standard was obtained from the National Institute of Biological Standards and Controls (NIBSC, Potters Bar). Standards were provided at a concentration of 10^6 International Units per ml (IU/ml). The HBV DNA standard was diluted in PBS to concentrations of 10^5, 10^4, 10^3, 10^2 and 10^1 IU/ml.

2.7.3: Extraction of DNA from patient serum samples and HBV standards – MagNA Pure robotic extraction

DNA was extracted robotically using the MagNA Pure system (Roche). 100 µl serum was diluted in PBS to a volume of 200 µl per sample and was extracted according to manufacturer's instruction. Extracted DNA was eluted to 100 µl elution buffer EB (Roche) and stored at -20°C.

2.7.4: Extraction of DNA from patient serum samples and HBV standards– Qiagen QIAamp DNA blood mini kit

The QIAamp DNA blood mini kit (Qiagen) is a spin column based kit, which incorporates sample lysis to release DNA. First DNA is adsorbed to a silica gel membrane column. A medium ethanol wash then removes protein and salt contaminants. The adsorbed DNA is then eluted in TE buffer and was either immediately used or stored at -20°C. DNA was extracted from 50 µl of patient serum sample in accordance with the manufacturer's instructions.

2.7.5: PCR Amplification of HBV small S gene

PCR primers were used which would amplify the entire small S gene in a nested PCR. Due to the overlapping HBV genome ORFs this protocol allowed amplification from *Pol* gene domains A, B and C, thus allowing antiviral resistance mutations to be identified as well as mutations of the S gene. The sequences of primers used for amplification of this region are shown in table 2.3 below:

Primer name	**Nucleotide Sequence**	**5' end***
Outer, sense Zo404	AGCCCTCAGGCTCAGGGCATA	1189
Outer, anti-sense C8602	AAACCCAGAAGACCCACAA	2332
Inner, sense POL1F	TCATCCTCAGGCCATGCAGT	1291
Inner, anti-sense M5877	ACACACTTTCCAATCAATAG	2305

Table 2.3 – Oligonucleotide primers used in nested PCR of HBV small S gene DNA

* All nucleotide numbers taken from the initiation triplet for core antigen = 1

** All oligonucleotide primers synthesised by Cruachem / Eurogentec

The first round mixture was as follows: 5 µl of extracted round product, 5 µl 10 X reaction buffer 5 µl 20 mM dNTPs, 2.5 µl 25 mM $MgCl_2$, 35 µl nuclease free water

(Promega) 2.5 µl each outer sense primer (M5877 and M5976) at 20 pmol /µl and 1 µl (1U) EXPAND DNA Polymerase (Roche). Cycling conditions for the first round PCR were as follows: initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 1 min followed by a final extension of 72°C for 5 min followed by a 4°C hold.

The second-round PCR mixture was as follows: 2 µl of first round product, 5 µl 10 X reaction buffer, 5 µl 20 mM dNTPs, 2.5 µl 25 mM $MgCl_2$, 35 µl nuclease-free water, 2.5 µl each inner primer (Zo404 and 186) at 20 pmol /µl. and 1 µl (1U) EXPAND DNA Polymerase. Cycling conditions for the second round PCR were as follows: initial denaturation at 95°C for 5 min followed by 35 cycles of 94°C for 30 seconds, 50° C for 30 seconds and 72°C for 1 min and then a final extension of 5 min and a 4°C hold.

PCR products were mixed with 2 µl 6 X Orange G loading dye (Promega) and loaded to a 2% agarose gel along with 1 µg of 1 kb DNA ladder to estimate the mass of the PCR product. Electrophoresis took place in a 1 X tris-borate-EDTA (TBE) buffer after which the gel was stained in a solution of 5 µg /ml ethidium bromide in TBE buffer. The bands were visualised using short wave UV transillumination and photographed using a darkroom Polaroid camera.

2.7.6: Purification of PCR products

PCR DNA was purified from solution using the GFX PCR purification kit (Amersham Biosciences), a spin column based kit that allows selective adsorption of DNA to a silica matrix whilst protein and salt contaminants are washed away by an ethanol wash. The DNA can then be eluted in nuclease-free water. 45 µl of PCR product from each

reaction was purified according to manufacturer's instructions, and the purified product was either used immediately in a sequencing reaction, or stored at -20°C.

2.7.7: Sequencing of PCR products

Purified PCR products were sequenced using 4 primers including the inner primers POL1F and M5877 used in the nested PCR reaction (table 2.3) thereby ensuring that the entire small S gene was sequenced by at least 2 primers reading in opposing directions. All primers used in the sequencing reactions are displayed in the table below (table 2.4). The sequencing reaction and analysis was completed under standard conditions for this study (section 2.1.7).

Primer name	**Nucleotide Sequence**	**5' end***
Sense POL1F	TCATCCTCAGGCCATGCAGT	1291
Sense H4074	TATCAAGGAATTCTGCCCGTTTGTCCT	1765
Anti-sense N2477	ACTGAGCCAAGAGAAACGGGCTGAG	1991
Anti-sense M5877	ACACACTTTCCAATCAATAG	2305

Table 2.4 – Primers used for sequencing the small surface gene PCR amplicon

* All nucleotide numbers taken from the initiation triplet for core antigen = 1.

** All oligonucleotide primers synthesised by Cruachem (Southampton, UK)

2.8.8: Predicting serotype from small surface gene sequence

Predicted serotype based on small S gene DNA sequence was calculated according to the algorithm shown in figure 2.5.

2.7.9: Phylogenetic Analysis of Sequence Data

Multiple alignments were made using Clustal W (Thompson *et al.,* 1994) which is a component of the Bio Edit software package (Hall, 1999). Pairwise alignments were made between nucleotide sequences to produce a guide tree estimating the final phylogenetic tree. Before a more extensive phylogenetic analysis was performed a Transition/ Transversion Ratio, base frequency and gamma distribution parameter α was estimated from the dataset. This was achieved using the program PUZZLE (Strimmer & von Haeseler 1996) Clustal W alignments were entered into the PHYLIP suite of programmes (Felsenstein, 1993), which allowed further analysis. The following programmes were used to analyse sequence data in PHYLIP: SEQBOOT, DNADIST, NEIGHBOR, DNAML, and CONSENSE. DNADIST creates a DNA matrix using one of three possible models of substitution; Jukes and Cantor (Jukes and Cantor 1969), Kimura (Kimura, 1980) and a maximum likelihood model (Felsenstein, 1981). The Kimura "2-parameter" method is used most often and assumes that independent changes occur at all sites with equal probability and allows for differences in transition and transversion rates. The output format of DNADIST is a matrix where genetic distances between sequences are roughly equivalent to percent nucleotide divergence values created using the MEGALIGN program (Lasergene –DNASTAR* 1997). The genetic distance values between species can be used in other programs to estimate branch lengths and draw trees. The Kimura "2-parameter" method was used to create distance matrixes from sequence data in this study. Transition/ transversion Ratios and gamma

distribution coefficient (calculated as 1/gamma parameter – estimated in PUZZLE) were entered into DNADIST. All resulting trees were thus corrected for these factors.

Trees were drawn using the NEIGHBOR program, which utilises either the neighbour-joining algorithm (Saitou and Nei, 1987) or the UPGMA method of clustering (Sneath and Sokal, 1973). NEIGHBOR creates successive clusters of closely related sequences and minimises branch lengths as the lineages join. No further rearrangements were made to the tree and because NEIGHBOR does not assume an evolutionary clock and the resulting tree is unrooted. Maximum Likelihood trees were drawn using DNAML using the CLUSTAL W alignment as starting material. As in the DNADIST analysis transition/ transversion ratios were entered for the dataset as well as the gamma distribution coefficient ($1/\alpha$) and empirical base frequencies thus correcting for these factors.

Maximum likelihood trees generated using DNAML were then compared to Neighbour joining trees generated in DNADIST and NEIGHBOUR to see if they generate a congruent phylogeny. A congruent phylogeny via the 2 methods would give confidence in the results from both. Confidence in the Neighbour joining result would allow bootstrapping analysis to proceed to asses the robustness of the phylogeny. Alignments were then analysed in SEQBOOT for 1000 multiple data sets, resampled from the input data set using random sampling methods with replacement. Trees generated in SEQBOOT were entered into the CONSENSE program and the branching patterns that occurred most frequently were reflected on the consensus tree. Values at each node indicate the amount of trees containing each branching pattern and could be used to interpret the robustness of the resulting tree. Although transition/ transversion ratios were entered into SEQBOOT analysis, gamma distribution coefficients were not, as

correcting for variation across the whole alignment was not appropriate, since bootstrap analysis interrogates sections of the alignment at a time and not the whole alignment. When the SEQBOOT programme is used, multiple trees are drawn from the NEIGHBOR program. CONSENSE then takes all these trees and creates a consensus tree to be viewed with the boostrap values inserted.

2.7.10: Molecular Epidemiological analysis

Once a phylogeny had been generated for the sequence data it was then correlated to risk factor data. Risk factor data could come from either one of two sources. When a patient's serum is sent to the SBVL at the Health Protection Agency Colindale, the referring microbiologist is asked to provide risk factor data on that patient from a questionnaire list. The list of factors is as follows: intravenous drug user, heterosexual contact, travelled to/ lived in endemic country, homo/ bi-sexual, transfusion recipient, health care worker, previous hepatitis, and contact of known hepatitis and 'other'. These data are often incomplete. However further risk factor data can be obtained via the Communicable Disease Surveillance Centre (CDSC) of the Health Protection Agency (Mary Ramsay, personal communication) via multiple ongoing epidemiological studies into acute incident HBV infection. The use of unique identifiers, i.e., patient's name, date of birth and location within the UK, which are available for patients identified through the reference laboratory screen or via CDSC studies allowed the addition of more risk factors to HBV sequences isolated from patients. The maximum likelihood phylogenetic tree was then annotated with the corresponding patient risk factor data and predicted serotype data using PowerPoint (Microsoft) allowing molecular epidemiological trends amongst those groups to be inferred

CHAPTER 3

OPTIMISATION OF TRANSFECTION VECTOR, CELL CULTURE AND ELISAs

3.1: Introduction

As sera were not available from patients that had been infected with the mutations that were to be studied, all HBsAg would have to be made *in vitro*. This would also ensure that all HBsAg was clonal, as mixed HBsAg populations may exist in patient sera due to quasispecies populations of infecting HBV. Firstly, a functional expression vector which would allow HBsAg production was to be constructed. Once established, suitable cell culture protocols had to be developed to maximise HBsAg production. Thereafter, ELISA assays which were suitably sensitive and specific had to be designed and validated. This chapter describes the optimisation of all these processes: vector construction, cell culture and ELISA design, up to the point at which HBsAg bearing mutations representative of drug resistant mutations could be assayed.

3.2: Construction of wild type and mutant eukaryotic transfection vectors containing the HBV small S gene

Reverse genetics has allowed the facile creation of genotypic mutants, using the known genomic sequence of an organism as the starting point. Such technology facilitates closer study of mutant phenotypic traits, thereby enabling the phenotype to be linked to a specifically created mutation. Reverse genetics methodology has allowed the production of HBsAg for both laboratory work and for vaccine (Valenzuela *et. al,* 1982). The reverse genetics approach contrasts with classical genetics, in which an organism with an unusual phenotype is first identified and the mutation that might have specified that phenotype then tracked back to a specific locus. This section (section 3.2) describes the first stages of a reverse genetics

experiment – the engineering of a vector capable of producing wild type HBsAg in eukaryotic cells and the subsequent mutagenesis of that vector to eventually express characteristics resembling mutant HBV that are resistant to antivirals.

3.2.2: PCR amplification DNA from the small S gene

The entire small S gene was amplified from the greater-than-full-length (1.3 times) parental genome $HBVCP_{IY}$ complete by a one-step PCR to generate a 794-bp amplicon (figure 3.1). The amplicon was suitable for restriction enzyme digestion and eventual cloning of the small S gene into vector the commercially available phagemid vector pBK-CMV

3.2.3: Vector pBK-CMV pre-cloning quality assay

To ensure vector quality and to assess damage that may have occurred through vector sub-cloning or long-term storage a single restriction digest using the enzyme *Kpn1* was undertaken. The observed difference in migration band between cut vector and uncut control (figure 3.1) was due to circularisation of the uncut control that migrated through the gel more rapidly due to its supercoiled structure. The vector was confirmed as circularised, an indication that the vector may not have suffered any damage. This experiment also allowed confirmation of vector mass as 4.5 kbp in length. A change in vector mass from the stated mass provided by the supplier could have indicated damage, alteration or that the incorrect vector was supplied, although this was not the case.

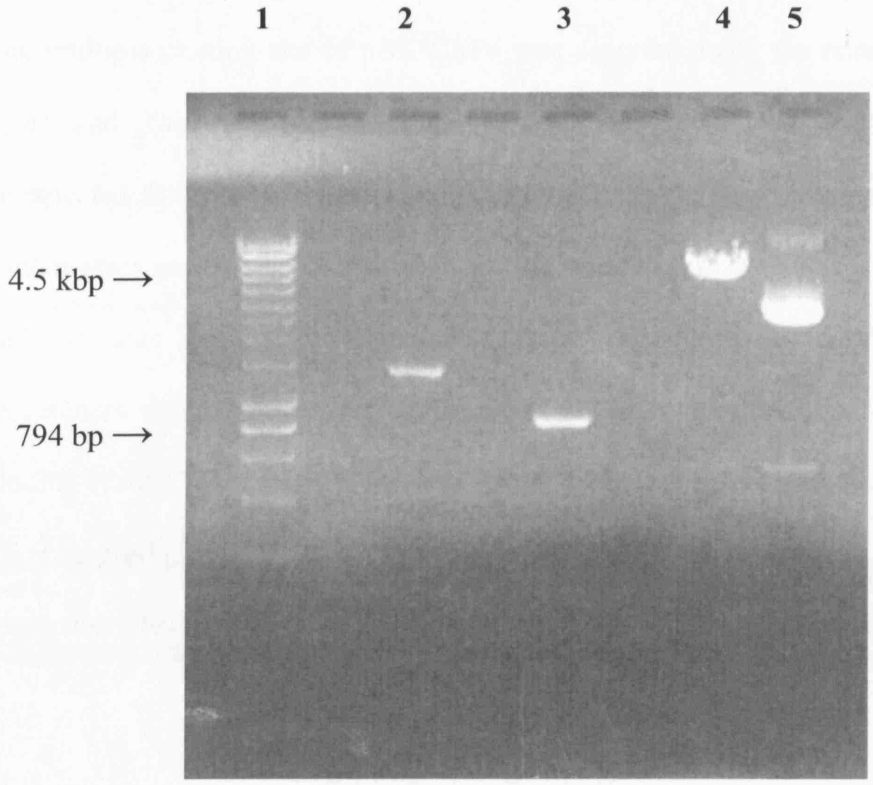


Figure 3.1 - Small S gene PCR product and pBK-CMV after *Kpn*1 digestion. Lane 1: Hyperladder 1 Molecular Weight Marker –; lane 2: product from large S gene [not used in this study] (1.2 kbp); lane 3: product from small S gene (794 bp); lane 4: pBK-CMV cut by *Kpn1* (4.5 kbp), Lane 5: pBK-CMV uncut by *Kpn1* (4.5 kbp).

3.2.4: Cloning of HBV small surface gene into pBK-CMV

The multiple cloning site of pBK-CMV was digested using the restriction enzymes *Kpn1* and *Xho1* (figure 3.2). This resulted in excision of a 50-bp band. An unexpected 600 bp band was also visible on the gel. This was suspected to be a contaminant and not of vector origin as the vector mass was correct post-digestion and the same band is known to appear in other experiments conducted by other researchers using the same reagents. It is not thought that its presence affected cloning results. The small S gene PCR amplicon was also digested with *Kpn1* and *Xho1* to produce a 721bp product (figure 3.3). This process created complementary restriction sites in each DNA component that would allow cloning to occur.

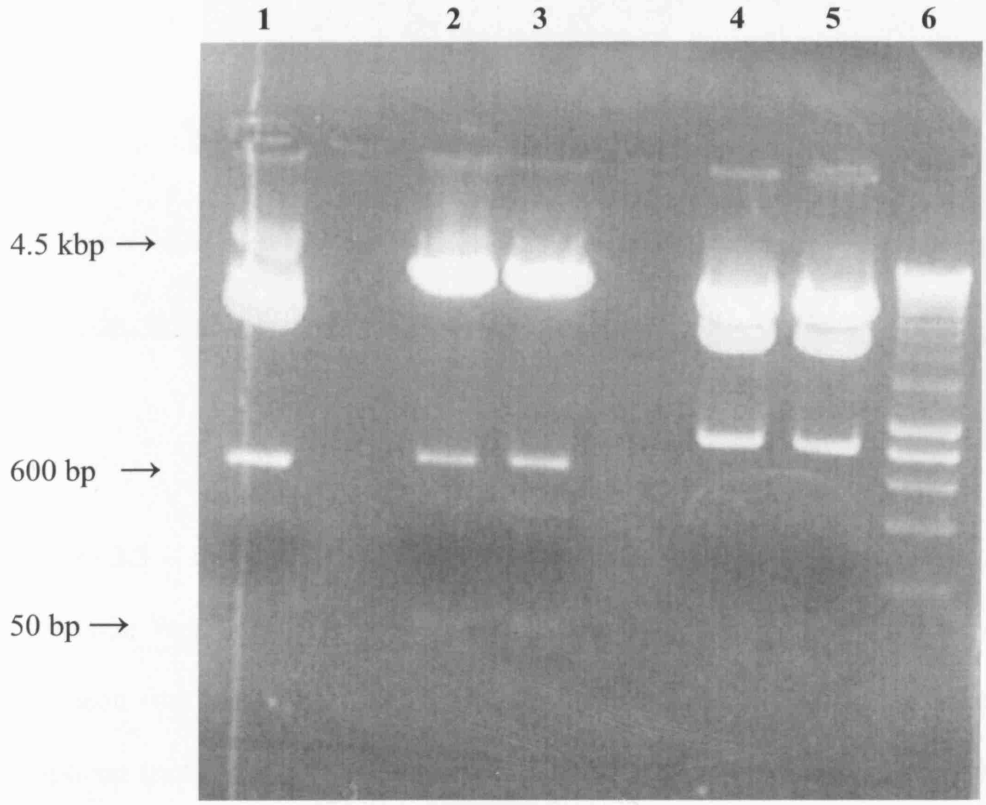


Figure 3.2 - *Kpn1* and *Xho1* digestion of pBK-CMV. Lanes 2 and 3 show duplicate data: *Kpn1* and *Xho1* digested pBK-CMV (the 4.5 kbp vector, 50-bp excision band and a 600-bp contaminant can be observed) Lane 6: Hyperladder 1 Molecular Weight Marker. [Lanes 1, 4 and 5 contain material not used in this study]

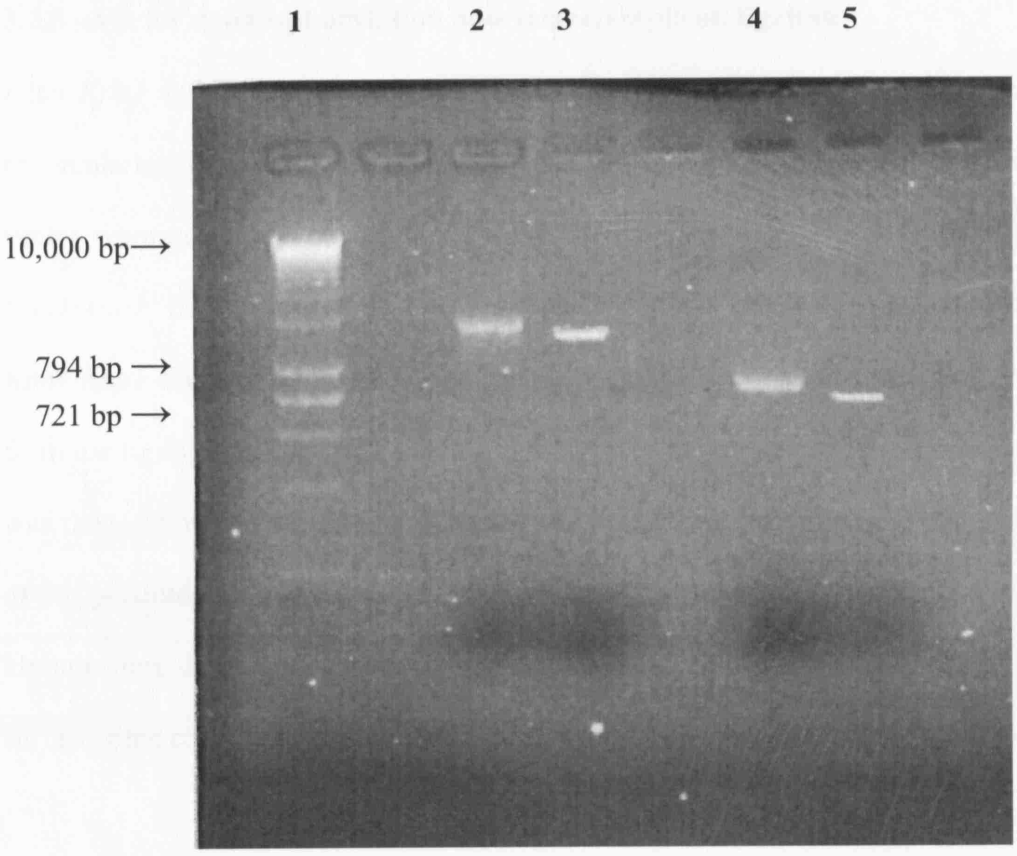


Figure 3.3 – *Kpn1* and *Xho1* digestion of small surface gene amplicon: Lane 1 Molecular Weight Marker (Hyperladder 1 -Bioline). Lane 2 undigested large S gene amplicon (not used in this study). Lane 3 *Kpn1* and *Xho1* digested large S gene amplicon (not used in this study). Lane 4 undigested 794 small S gene amplicon. Lane 5 *Kpn1* and *Xho1* digested 721-bp small S gene amplicon.

3.2.5 - Vector dephosphorylation and vector/amplicon ligation

After *Kpn1* and *Xho1* digestion, vector pBK-CMV was dephosphorylated to prevent re-circularisation (although this was unlikely to occur as the two cut sites left in the vector were not complementary). A ligation reaction was then performed with the *Kpn1/Xho1* digested and dephosphorylated vector pBK-CMV and the 721-bp *Kpn1/Xho1* digested PCR amplicon that contained the small S gene. The product from the ligation reaction was then used to transform competent cells. Plasmid DNA was then extracted from the transformed cells via Qiagen miniprep. Electrophoresis of this plasmid demonstrated that the ligation product plasmid mass was just over 5.2 kb indicating that the ligation was successful and that pBK-CMV contained the small surface gene cassette within the MCS (figure 3.4).

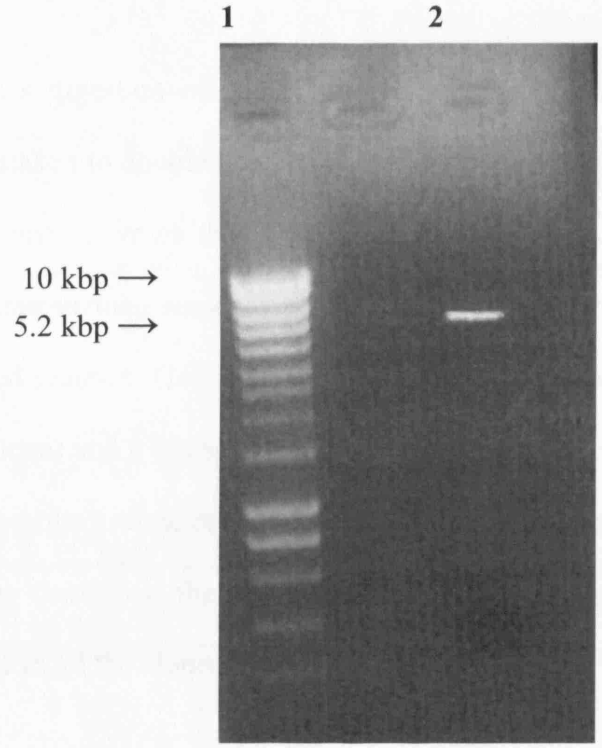


Figure 3.4 – Electrophoresis of ligation product: Lane 1, Molecular Weight Marker (Hyperladder 1 – Bioline). Lane 2, 5.2 Kbp Plasmid isolated from cells transfected with *Xho1*/*Kpn1* treated small S amplicon + *Xho1*/*Kpn1* treated pBK-CMV ligation product.

3.2.6: Confirmation of small surface gene cassette insertion within vector

Further digestion of the vector containing the small S gene cassette was then undertaken to double check that the ligation had definitely been successful. By using the same enzymes that were used to clone the cassette into vector it should be possible to then remove the inserted small surface gene cassette from the newly ligated product. This would be expected to result in a 721-bp band (representing the amplicon) and a 4518-bp band (representing the vector) compared to a 5239-bp band (representing amplicon and vector). Using this approach DNA extracted from 8 clones confirmed the presence of the small S gene insert within the pBK-CMV vector in all the clones (figure 3.5 and figure 3.6).

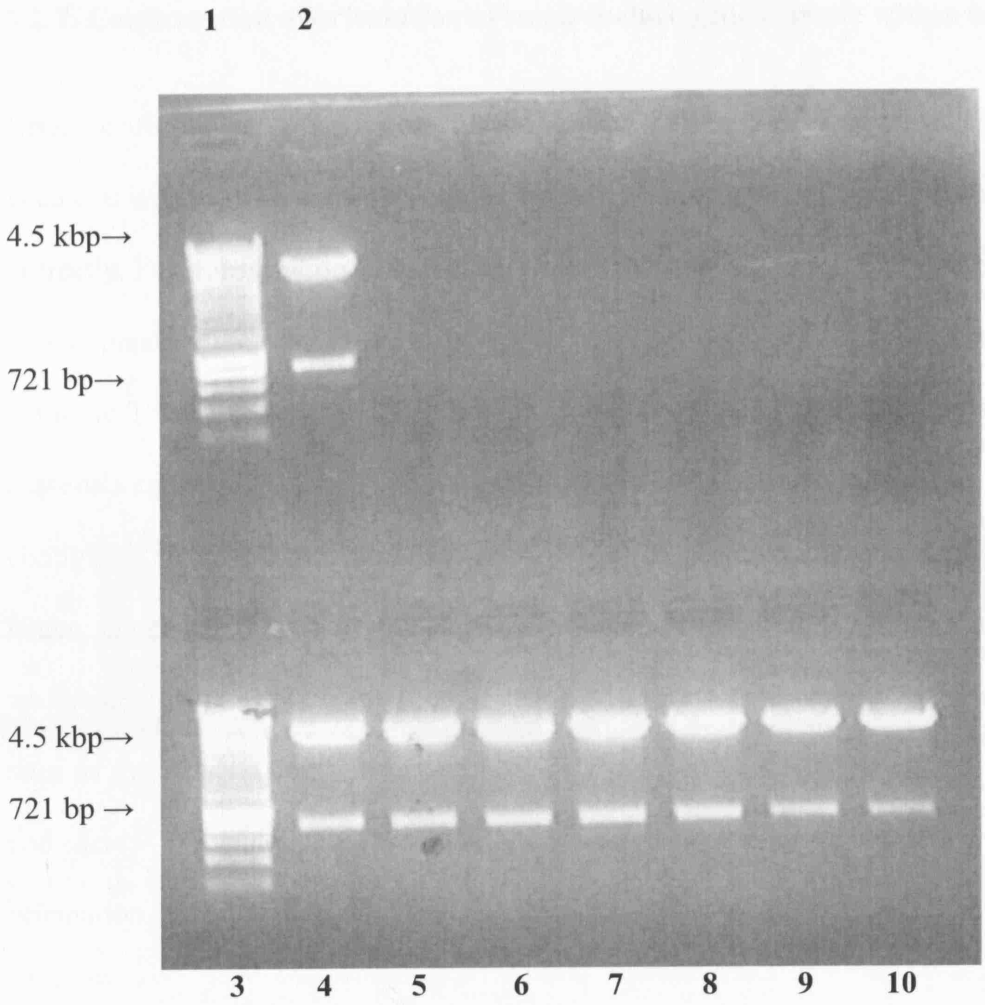


Figure 3.5 - Digestion with *Kpn1 and Xho1* of plasmid DNA obtained from cells transformed with pBK-CMV which potentially contained small S gene cassette. Lanes 1 and 3: Molecular Weight Marker (Hyperladder 1- Bioline), lanes 2 and 4-10: *Kpn1* and *Xho1* treated plasmids.

3.2.7: Confirmation of orientation of small surface gene cassette within vector

Upon confirmation of the successful cloning of the small S gene cassette into the vector, it was next necessary to ensure that the cassette was orientated in the vector correctly. For transcription of the inserted gene to occur under the CMV IE promoter it is imperative that the 5' end of the gene (the start codon) be placed downstream from the 3' end of the CMV IE promoter. There exists the potential to clone a gene cassette into the pBK-CMV vector in the incorrect orientation (i.e. 3' end of the gene contiguous to the 3' end of the upstream CMV IE promoter) within the MCS and hence under the SV40 promoter. This situation can arise if cloning has been undertaken using only one restriction enzyme to create complementary 'sticky end' sites as the cassette could insert either way. However, I used two enzymes *BamH1* and *Xcm1* to create complementary sites which would ensure that the correct orientation is maintained.

The orientation assay was performed by *BamH1* and *Xcm1* digestions of the plasmids containing the small S gene cassette. If the cassette was contained within the vector in the correct orientation, a 590-bp fragment would be observed upon electrophoresis of digest products, but if the orientation was incorrect a 770-bp fragment would be visible.

Figure 3.8 shows the outcome of a typical digestion experiment. The 590-bp fragment was seen in 3 of 6 plasmids digested, the other 3 presumed to contain the cassette in the incorrect orientation. Any plasmids containing the small S gene in the correct orientation were designated pBK-CMV[smallS]. The first 3 pBK-

CMV[smallS] clones identified in this assay were designated R1, R2 and R3 which contained the wild type gene originating from the parental genome $HBVCP_{IY}$.

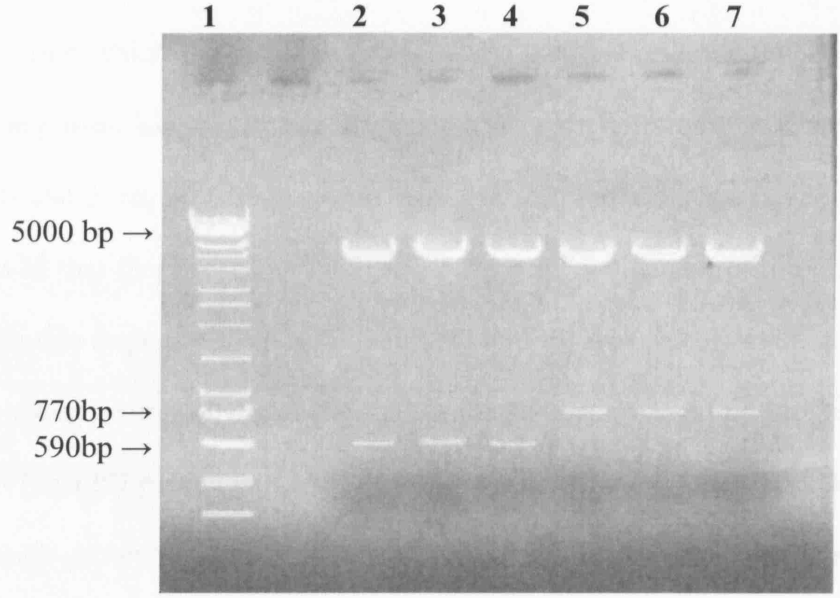


Figure 3.6 – Confirmation of the correct orientation of the small surface gene cassette within pBK-CMV by *BamH1* and *Xcm1* digestion: Lane 1: 1 kb DNA Ladder Molecular Weight Marker ; lanes 2, 3 and 4: Vector plus 590-bp band (correct orientation); lanes 5, 6 and 7: Vector plus 770 bp band (incorrect orientation).

3.2.8: Nucleotide sequencing of small S gene cassette inserted into vector

It was then necessary to sequence vector pBK-CMV[smallS] plasmids R1, R2 and R3. This was done using the T3 and T7 promoter sites, which flank each side of the MCS into which the small S gene cassette had been cloned, as sequencing primer binding sites. The nucleotide sequence and predicted amino acid sequence for both the S and P reading frames (appendix 2.1, 2.2 and 2.3 respectively – "wild type") showed that there were no nucleotide changes. Sequencing also confirmed that the nucleotide sequence for plasmids R1, R2 and R3 were identical. The generation of a neighbour joining phylogenetic tree in the DNAstar Megalign program using pBK-CMV[smallS] plasmids R1, R2 and R3 against standard GenBank HBV sequences for each genotype (A to H) confirmed the small S gene as belonging to genotype A HBV. The predicted amino acid sequences for R1, R2 and R3 specify serotype *adw2*. Confirmation of the cassette nucleotide sequence thus allowed eukaryotic cell transfection work to proceed in order to determine if the vector was viable for expression of small S protein.

3.2.9: Initial transfection of eukaryotic cells with pBK-CMV[smallS]

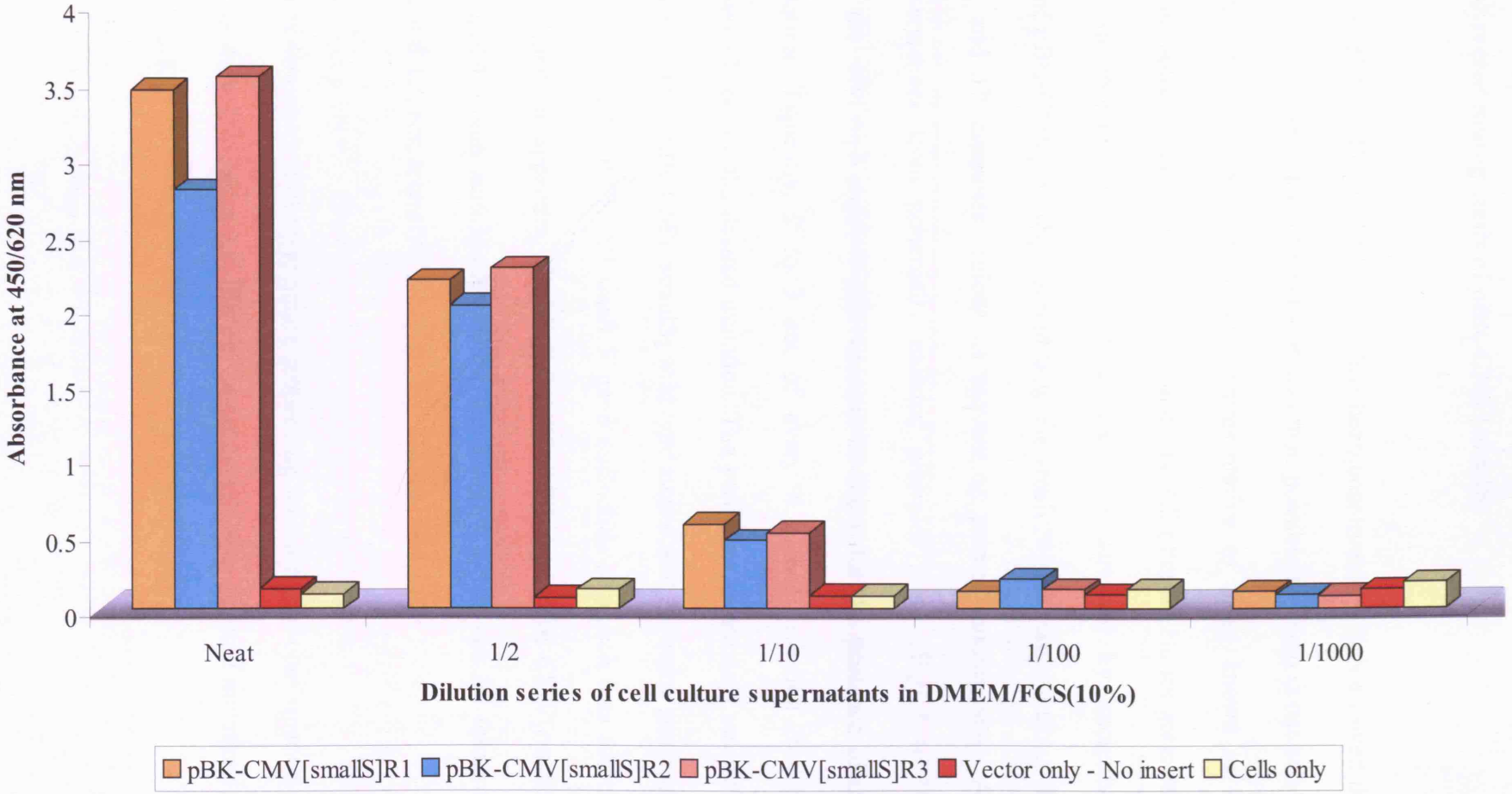
In order to test the ability of pBK-CMV[smallS] to transform cells and produce HBsAg, Cos-7 cells were transfected with pBK-CMV[smallS] R1, R2 and R3. Although the final aim of the study was to use a cell line of human hepatic origin, such as HepG2 cells, Cos-7 cells were used for vector assessment because of their availability, ease of culturing, ease of transfection and propensity to express HBsAg upon transfection (Mangold *et. al.,* 1995). Cells were transiently transfected with pBK-CMV[smallS] using lipofectamine 2000. Supernatants were harvested and clarified 2 days post transfection.

The initial transfection of Cos-7 was considered successful as all cell cultures transfected by the three plasmids R1, R2 and R3 were observed to secrete HBsAg detectable by the Ge34/36 HBsAg ELISA of cell culture supernatant fluid. HBsAg could be detected up to a 1:10 dilution of the fluid (figure 3.7). The absorbances obtained at neat concentration were 3.77 for R1, 2.78 for R2 and 3.53 for R3. These values are high as a value of 3.9 denotes saturation. However, Ge34/36 is known to be a very sensitive assay with peak sensitivity (i.e. absorbance saturation) of 3 ng/ml HBsAg (R Tedder, personal communication). The results for R1-R3 approach that peak. Nonetheless, these values are low when compared to levels of HBsAg found in patients infected with HBV and particularly when compared to levels of HBsAg achieved in other studies using similar vectors to produce HBsAg (Jeantet *et. al.,* 2004). The low levels of HBsAg achieved in this experiment would be problematic if used as the final means of production of HBsAg, as large volumes of cell culture supernatant would be required to fully assay each mutant protein. It was of concern that cell expressed wild type HBsAg was unable to reach peak absorbancy in the ELISAs which were to be used to assay HBsAg reactivity. Under such conditions of

low HBsAg concentration there may be insufficient dynamic range within the assay to discriminate wild type HBsAg reactivity from less reactive mutant HBsAg.

There are many possible solutions to improve this performance. These include: placing the cassette under a different promoter in pBK-CMV such as the SV40 promoter; change of vector to another CMV IE vector; change of vector to another vector containing a different promoter e.g. the 'strong' chicken actin promoter found in many new commercially available vectors; optimisation of transfection efficiency; stabilisation of the cell line; using other cell types; optimisation of ELISA performance; and concentration of HBsAg from low yield cell culture. The rationale for changing vector was not convincing given that other groups had successfully used the same CMV IE promoter in very similar vectors for HBsAg production (Jeantet *et. al.,* 2004). As there was ample opportunity to improve expression performance through transfection, culturing and ELISA sensitivity optimisation, it was felt that these were the steps to make the necessary improvements. The need to re-clone the cassette or to resort to large-scale cell culture would thus be obviated Hence work on site directed mutagenesis of pBK-CMV[smallS] proceeded.

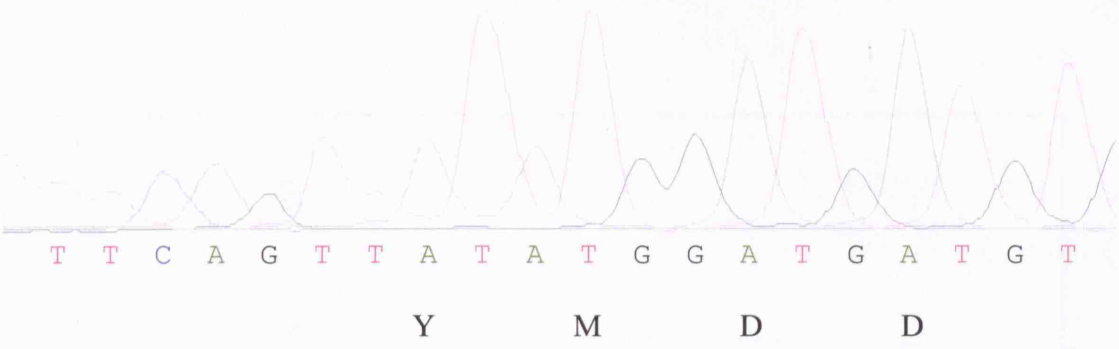
Figure 3.7 - Initial transfection of Cos-7 cells with pBK-CMV[smallS] R1, R2 and R3: results of Murex Ge34/36 HBsAg ELISA analysis of cell culture supernatants



3.2.10: Site directed mutagenesis of pBK-CMV[smallS]

As a wild type pBK-CMV[smallS] vector had been constructed which allowed the production of HBsAg in eukaryotic cells, it was then possible to begin constructing vectors containing small S gene mutants representative of those known to be associated with antiviral induced resistance mutations. All plasmid clones generated through the site-directed mutagenesis procedure were screened by nucleotide sequencing of pBK-CMV[smallS] inserted with the small S gene cassette using the flanking T3 and T7 promoter regions as sequencing primer binding sites. All nucleotide sequences from potentially mutated plasmids were aligned to the sequence of the wild type small S gene cassette to check for the presence of the desired mutation. Typically, 2 to 3 out of every 4 clones screened through sequencing would contain the desired mutation. The predicted amino acid sequence was then aligned to the pBK-CMV[smallS] wild type amino acid sequence generated from both the S and P ORFs. All small S gene nucleotide sequence data for the mutants are found in appendix 2.1 aligned with wild type pBK-CMV[smallS] cassette. Predicted amino acid sequences for the S and P genes can be found in appendix 2.2 and 2.3, respectively.

An example of data observed in nucleotide sequencing screening for confirmation of mutagenesis is shown in figure 3.8. The list of successful site-directed mutations is shown in table 3.1.



Wild Type sequence

rtM204V/sI195M mutant sequence

Figure 3.8 – Example of raw chromatogram nucleotide sequence data in post site directed mutagenesis screening of plasmid cassettes. (Edited data for all mutants in appendices 2.1-2.3)

Mutant	Antiviral associated with mutation	Reference
rtM204V/sI195M	lamivudine	Ling *et al.*, 1996; Tipples *et al.*, 1996
rtM204I/sW196S	lamivudine	Ling *et al.*, 1996
rtM204I/sW196L	lamivudine	Ling *et al.*, 1996
rtL180M/sSilent	lamivudine	Ling and Harrison, 1999
rtL180M/sSilent + rtM204V/sI195M	lamivudine	Ling and Harrison, 1999
rtV173L/sE164D	lamivudine	Ogata *et al.*, 1999; Delaney *et al.*, 2003
rtV173L/sE164D + rtM204V/sI195M	lamivudine	Delaney *et al.*, 2003
rtT128N/sP120T	lamivudine	Torresi *et al.*, 2002b
rtT128N/sP120T + rtM204V/sI195M	lamivudine	Torresi *et al.*, 2002b
rtSilent/sD144E	lamivudine	Torresi *et al.*, 2002b
rtSilent/sD144E + rtM204V/sI195M	lamivudine	Torresi *et al.*, 2002b
rtR153Q/sG145R	lamivudine	Torresi *et al.*, 2002b
rtR153Q/sG145R + rtM204V/sI195M	lamivudine	Torresi *et al.*, 2002b
rtR153K/sD144EsG145R	lamivudine	Torresi *et al.*, 2002b
rtR153K/sD144EsG145R + rtM204V/sI195M	lamivudine	Torresi *et al.*, 2002b
rtF166L/sF158Y	lamivudine	Torresi *et al.*, 2002a
rtF166L/sF158Y + rtM204V/sI195M	lamivudine	Torresi *et al.*, 2002a
rtA181T/sW172STOP	adefovir	Hadziyannis *et al.*, 2005
rtA181V/sS173F	adefovir	Hadziyannis *et al.*, 2005
rtI169T/sF161L	entecavir	Tenney *et al.*, 2004
rtT184S/sL176V	entecavir	Tenney *et al.*, 2004
rtS202I/sV194F	entecavir	Tenney *et al.*, 2004
rtS202I/sV194F + rtT184S/sL176V	entecavir	Tenney *et al.*, 2004
rtS202I/sV194F + rtT184S/sL176V + rtI169T/sF161L	entecavir	Tenney *et al.*, 2004

Table 3.1 – List of mutations in S gene generated via site directed mutagenesis .

3.2.11: Vector construction overview

A vector capable of inducing expression of wild type HBV small S gene protein was successfully engineered. HBsAg expression levels, though not formally quantified, were suspected to be low to moderate in comparison to other such studies that used similar commercially available CMV IE promoter vectors (Jeantet *et. al.,* 2004). However the expression levels were sufficient to permit HBsAg ELISAs to be conducted.

3.3 Optimisation of eukaryotic cell pBK-CMV[smallS] transfection and cell culture systems to improve yield of secreted HBsAg

The levels of HBsAg shed from Cos-7 cells following their transfection with the newly constructed pBK-CMV[smallS] were lower than expected. To use such a system to generate HBsAg for the intended study would be problematic. Large volumes of cell culture supernatant would be required which is challenging to scale up. Furthermore, if wild type HBsAg could not be generated in levels sufficient to reach peak absorbances in the ELISAs, mutant HBsAg reactivities may be difficult to distinguish from wild type reactivities, as the differences observed between the wild type reactivity and that of a less reactive mutant may be too little

.

The problem of HBsAg level can be approached at different stages: At the most fundamental level, the vector can be changed or altered. Next, improvements to transfection efficiency can be made, then changes of culture conditions and, finally, the protein can be purified and concentrated. As pBK-CMV[smallS] is functional in Cos-7 cells following transfection, there was potential to improve the transfection and cell culture stages without the need to alter the vector. This section (section 3.3) deals with experiments undertaken to raise higher HBsAg levels from Cos-7 and other eukaryotic cells.

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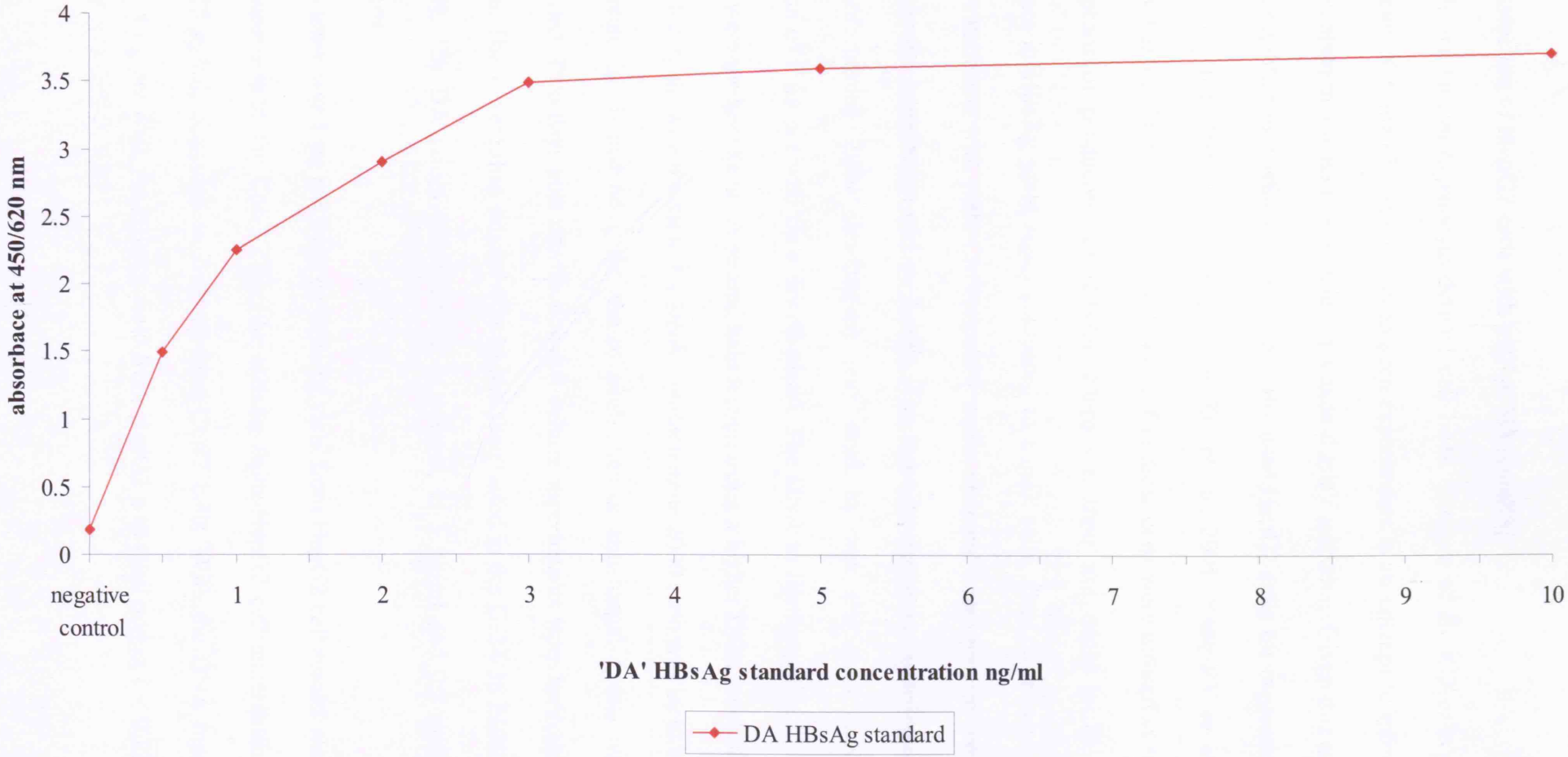
3.3.1: Quantification of HBsAg

The upper limit of detection of HBsAg in the Ge34/36 ELISA was considered to be about 3 ng/ml HBsAg (R.Tedder, personal communication). To determine which cell culture transfection protocol would be selected, a quantification assay for HBsAg using Ge34/36 ELISA was adapted. The 'DA' serum was used as an HBsAg standard. The standard was diluted in DMEM/FCS (10%) to provide 10 ng/ml, 5 ng/ml, 3 ng/ml, 2 ng/ml, 1 ng/ml and 0.5 ng/ml HBsAg concentrations, and assayed in the Ge34/36 ELISA in quadruplicates. From the mean results obtained a standard curve was drawn (figure 3.9) which was then used to quantify HBsAg thereafter. The curve did indeed plateau at 3 ng/ml (at an absorbance of 3.492) as anticipated. Although the results for 5 ng/ml and 10 ng/ml are slightly higher, at 3.591 and 3.731 respectively, it would be difficult to use such a slight difference as a basis for quantification, stressing the need in future experiments to always assay one sample at a 1 in 10 dilution.

Figure 3.9 - HBsAg concentration vs. absorbance in Murex Ge34/36 HBsAg ELISA using quantified 'DA' HBsAg standard

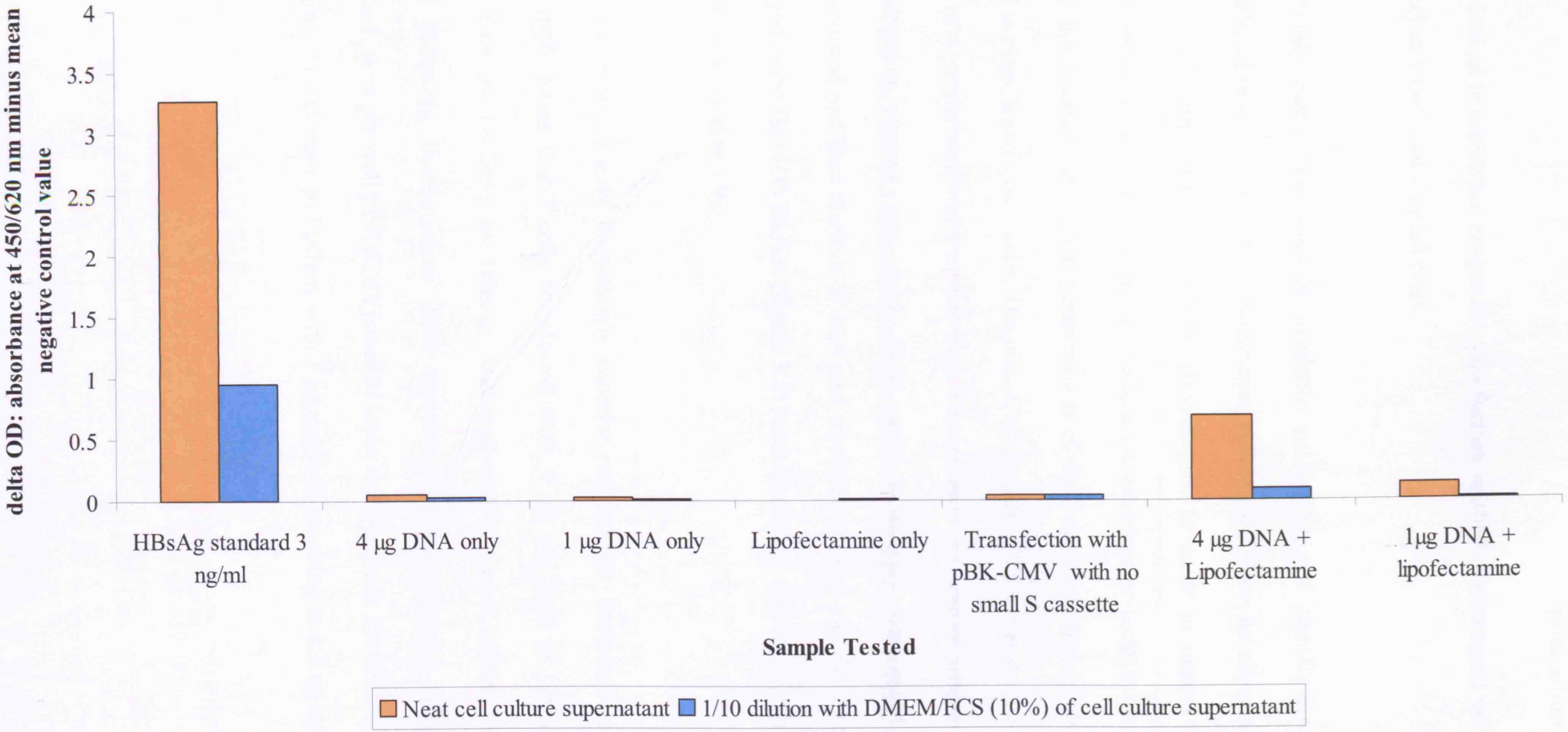


3.3.2: Transfection of HepG2 cells with pBK-CMV[smallS]

HepG2 cells are human hepatocyte derived cell lines. The use of HepG2 cells or other cell lines of human hepatic origin in such experiments is an attempt to mirror the cell population that would be infected in a natural HBV infection. Given this and the wealth of publications which have successfully used HepG2 cells for expression of HBsAg and other HBV proteins (e.g. Yang ZG *et. al.,* 2005, Yang RY *et. al.,* 2003, Oon CJ *et. al.,* 2002 and Heijtink *et. al.,* 2001) these cells were utilised as the preferred means of production of HBsAg. Moreover, their use could result in improvements in HBsAg yield when compared to Cos-7 cells. HepG2 cells were transiently transfected with pBK-CMV[smallS] under identical conditions to those used in the initial transfection of Cos-7 cells. The same lipofectamine transfection reagent lipofectamine 2000 (Invitrogen) was used as was the same DNA concentration of 1 μg per well (in a 6 well plate). The DNA to lipofectamine 2000 ratio of 3:1 were the kept same. A second transfection using a higher DNA level of 4 μg per well but again with the same 3:1 DNA: lipofectamine 2000 ratio was included for comparison. In all instances, the use of antibiotics or anti-fungal agents was strictly avoided. Two days post transfection cell culture supernatants were harvested and clarified. These were then diluted 1 in 10 and then tested in the Ge34/36 ELISA (figure 3.10). The DA serum was included as control, at 3 ng/ml and 0.3 ng/ml concentrations.

When DNA input was 1 μg per well, the HBsAg yield from HepG2 cell results were poor in comparison to the Cos-7 cells, the HBsAg from HepG2 cell supernatants being <0.125 ng/ml, compared to 2 ng/ml from Cos-7 cells. With the DNA input increased to 4 μg per well, the HBsAg yield from HepG2 was even poorer (< 0.125 ng/ml).

Figure 3.10 - Initial transfection of HepG2 cells with wild type pBK-CMV[smallS] : Delta OD's of cell culture supernatants and DA HBsAg standard in Ge34/36 ELISA

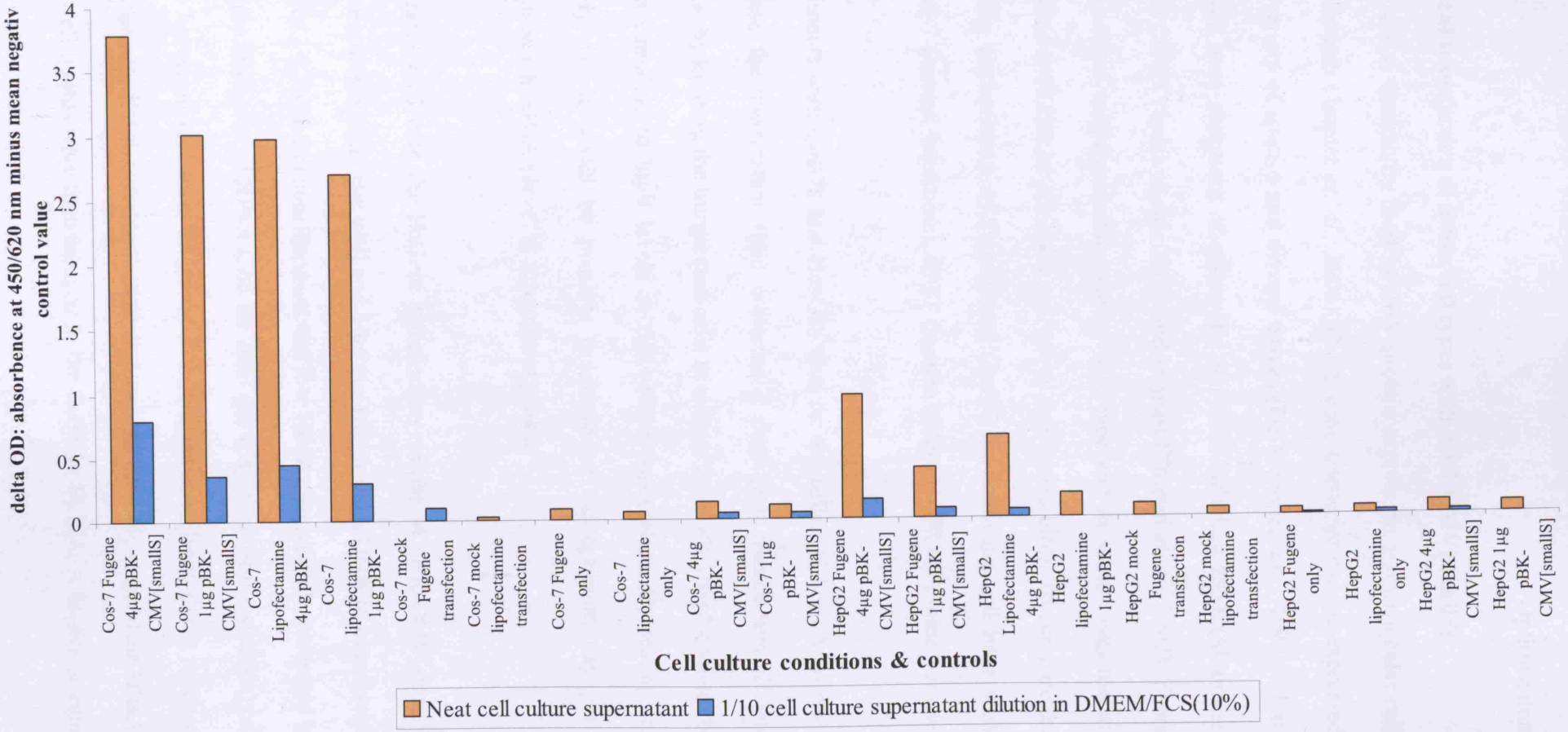


3.4.2: Comparison of transfection reagents - Lipofectamine2000 (Invitrogen) vs. FuGene (Roche) in Cos-7 and HepG2 cells.

There are currently many commercially available eukaryotic cell transfection reagents available, all promising very high transfection efficiencies. Thus far all cells had been transfected with lipofectamine 2000 (Invitrogen). In order to improve transfection efficiency, the transfection reagent FuGene (Roche) was investigated as an alternative to lipofectamine 2000. The performances of the 2 transfection reagents were compared in HepG2 and Cos-7 cells. The pBK-CMV[smallS] DNA was used at concentrations of 4 μg per well and 1 μg per well. The standard 3:1 weight ratio of DNA to transfection reagent was used. Cells were transiently transfected, supernatants harvested and then clarified 2 days post transfection. The supernatants were then assayed in the Ge34/36 ELISA (figure 3.11) and quantified against the DA HBsAg standard curve (figure 3.9).

The FuGene-based protocol gave consistently superior performance compared to lipofectamine 2000. From Cos-7 cells transfected with 4 μg per well of pBK-CMV[smallS] DNA and Fu-Gene, the HBsAg yield approached 3 ng/ml, compared with 2 ng/ml following lipofectamine 2000 transfection. From HepG2 cells transfected with 4 μg of per well pBK-CMV[smallS] DNA and Fu-Gene, the HBsAg yield was 3 ng/ml. Transfection in FuGene with 1 μg per well resulting in 1.5 ng/ml HBsAg.

Figure 3.11- Comparision of FuGene (Roche) and lipofectamine LF2000 (Invitrogen) transfection reagents in Cos-7 and HepG2 cell lines using Murex Ge34/36 HBsAg ELISA



3.4.3: Transient transfection of other cell types with pBK-CMV[smallS]

Huh-7 cells are also commonly used in HBV protein expression and molecular cell biology experiments (Jeantet *et. al.,* 2004) The S gene transcript can be expressed from a wide variety of species and diverse tissues (De Meyer *et. al.,.*, 1997). Thus, HBsAg has also been expressed in eukaryotic cell lines including 293T (Moore *et. al.,* 2005), and CHO (Vianna *et. al.,* 2003) and in yeast (Toressi *et. al.,* 2002). These studies all produced conformationaly stable, wild type and in some cases mutant HBsAg suitable for ELISA or antibody analysis (e.g. Western blot). To compare the HBsAg-yielding performance of HepG2 and Cos-7, the following cell lines were analysed: Huh-7 (human hepatoma), 293T (human kidney), CHO (Chinese hamster ovary).

In the experiments with Cos-7s and HepG2s thus far the cells had been transiently transfected and the supernatant fluid collected 2 days post transfection. It was postulated that by keeping the transfected cells in culture for 1 wk, HBsAg would be allowed to accumulate to high levels in the culture supernatant. By testing the cultures every day it would be possible to determine the optimum day post-transfection in which HBsAg level in supernatant peaks.

Cells were transfected with the FuGene transfection reagent at pBK-CMV[smallS] DNA concentrations of 4 μg per well and 1 μg per well using a DNA to transfection reagent ratio of 3:1. With the consideration that low HBsAg secretion levels might be due to secretion inhibition of HBsAg, on the final day (day six post-transfection) cell pellets were harvested and then subjected to non-denaturing lysis. (This mode of lysis was to protect the membrane bound and conformationaly dependant HBsAg molecules). Cell lysates were then assayed in the Ge34/36 ELISA. A higher or equal

concentration of HBsAg in cells compared to HBsAg concentration in cell culture supernatants may indicate that secretion inhibition was occurring (Jeantet *et. al.,* 2004).

With cell supernatants, it was found that Huh-7, 293T and HepG2 cells yielded small amounts of HBsAg throughout the entire time course. Cos-7 cells yielded comparatively moderate amounts of about 1 ng/ml HBsAg peaking at day 6 post-transfection, and CHO cells yielded the most HBsAg. CHO cells gave an initially high peak on day 3 of about 2 ng/ml , then declined but thereafter peaked to approach 3 ng/ml at day 5 (figures 3.12-3.16).

With the day 6 HBsAg yields from supernatants combined with lysates, no changes in the lysate to supernatant HBsAg ratios were observed. These observations accord with the data of Jeantet *et. al.,* 2004 showing that secretion inhibition was not occurring.

Figure 3.12- Huh-7 transfection with wild type pBK-CMV[smallS]: results of Murex Ge34/36 HBsAg ELISA analysis of cell culture supernatant days 1-6 post transfection and cell pellet lysates day 6 post transfection

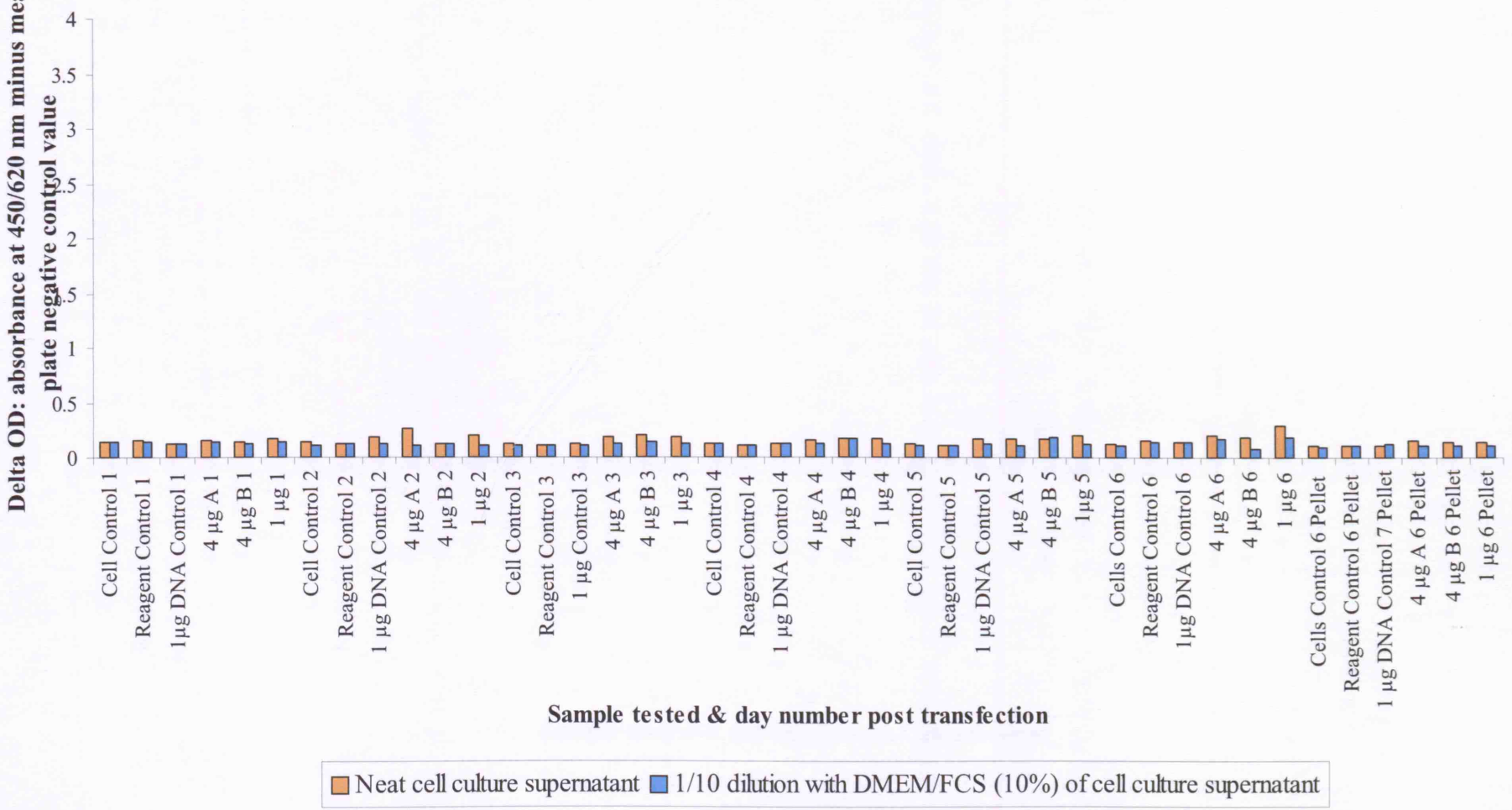


Figure 3.13 - 293T transfection with wild type pBK-CMV[smallS]: results of Murex Ge34/36 HBsAg ELISA analysis of cell culture supernatant days 1-6 post transfection and cell pellet lysates day 6 post transfection

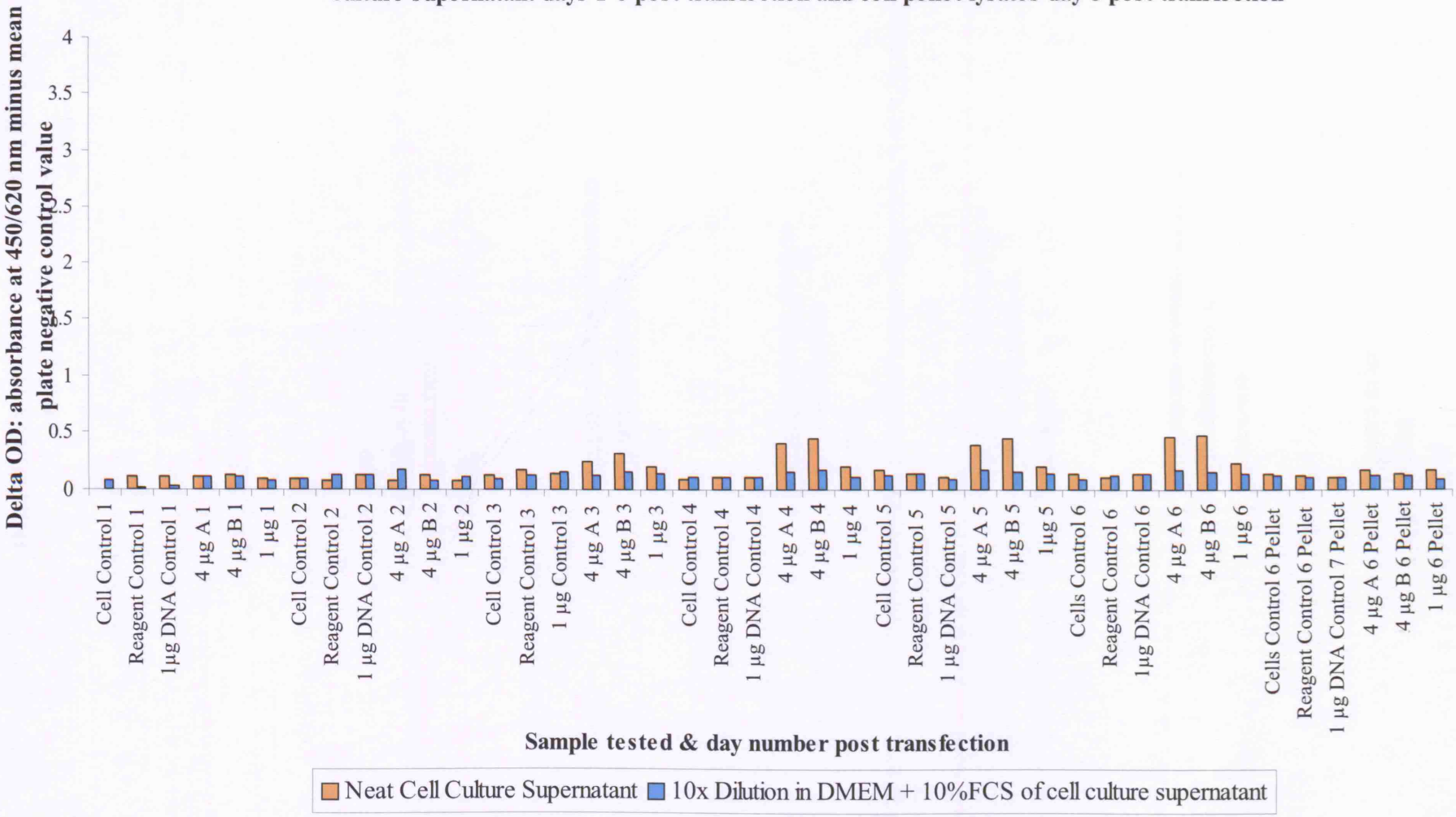


Figure 3.14 - CHO transfection with wild type pBK-CMV[smallS]: results of Murex Ge34/36 HBsAg ELISA analysis of cell culture supernatant days 1-6 post transfection and cell pellet lysates day 6 post transfection

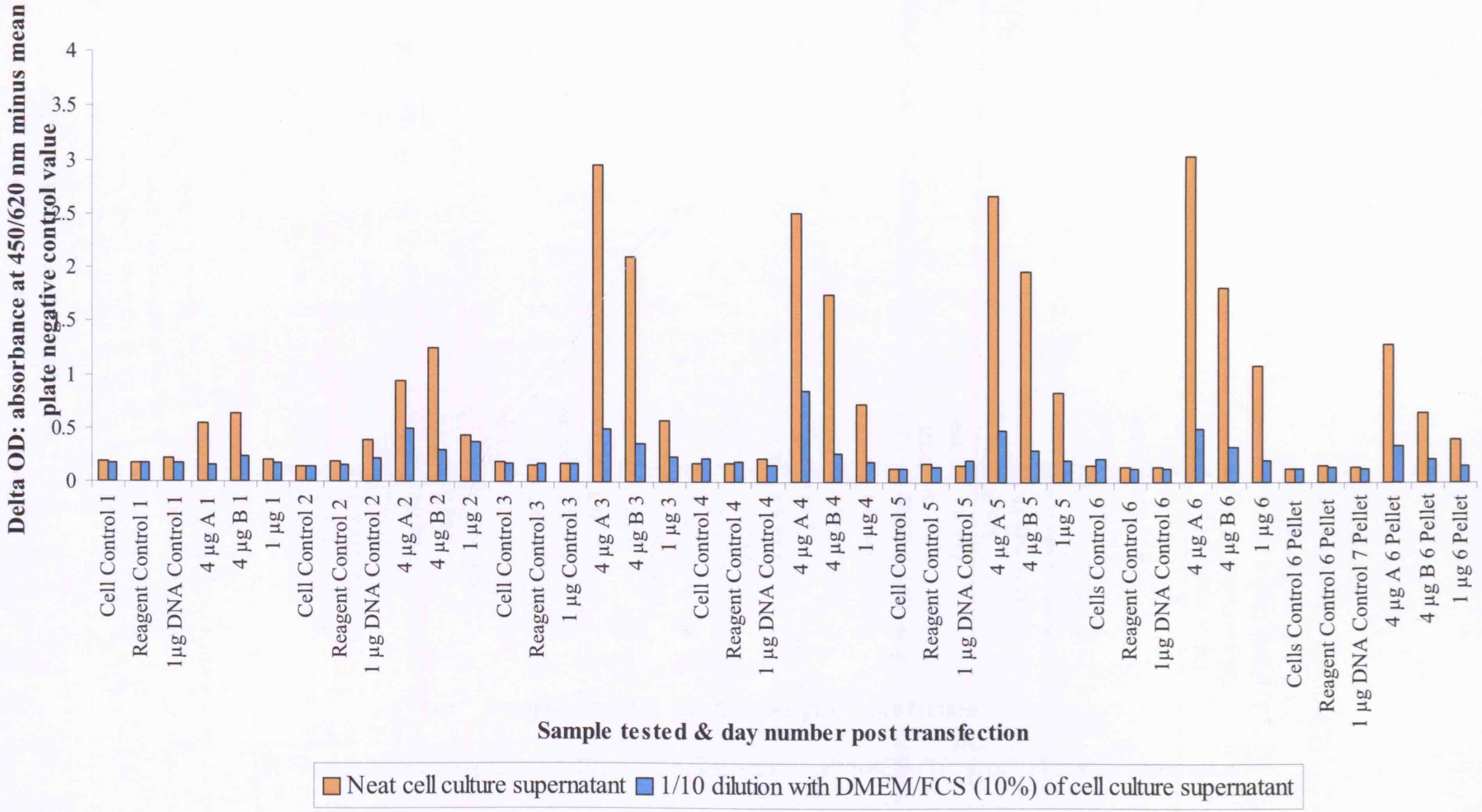


Figure 3.15 - HepG2 transfection with wild type pBK-CMV[smallS]: results of Murex Ge34/36 HBsAg ELISA analysis of cell culture supernatant days 1-6 post transfection and cell pellet lysates day 6 post transfection

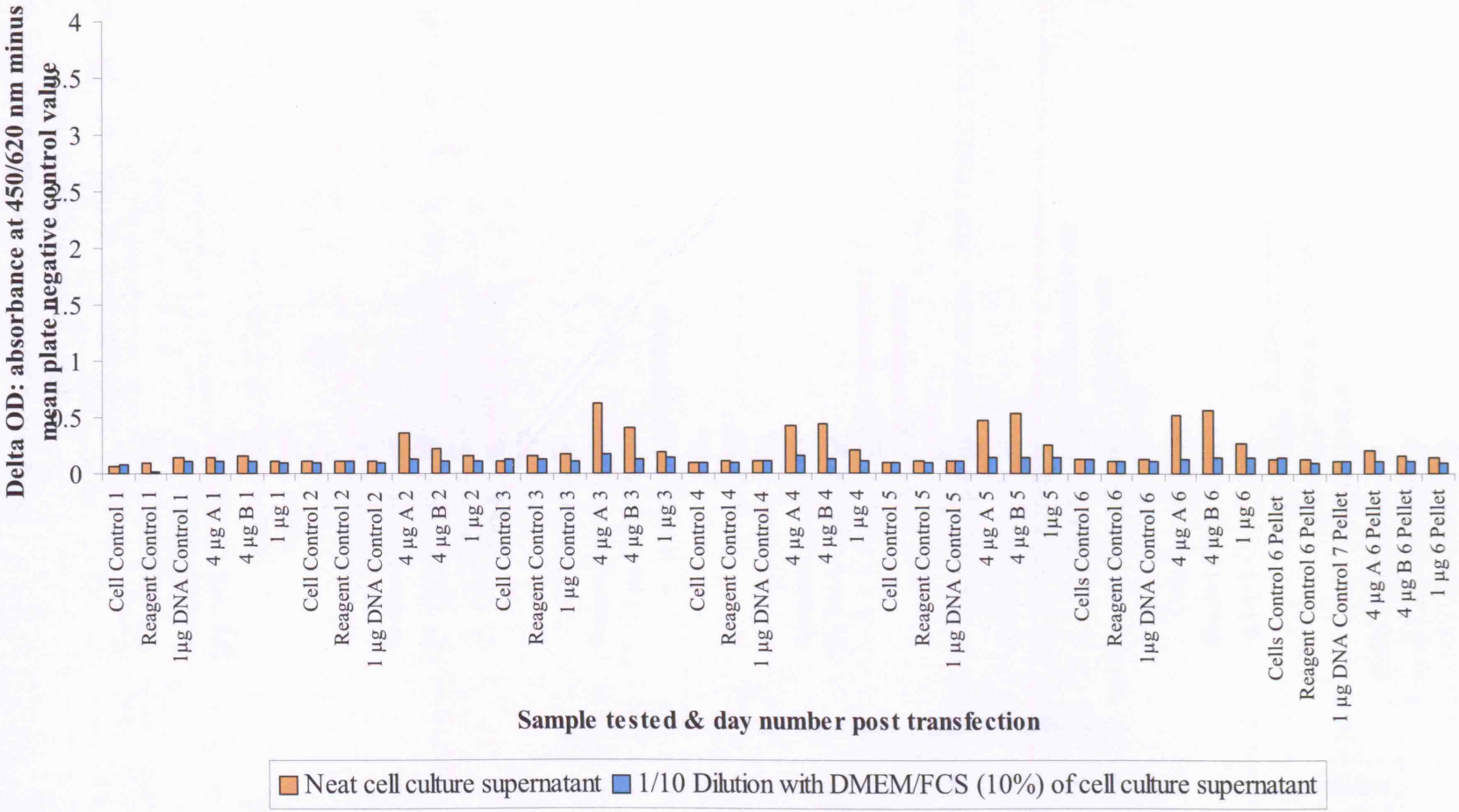
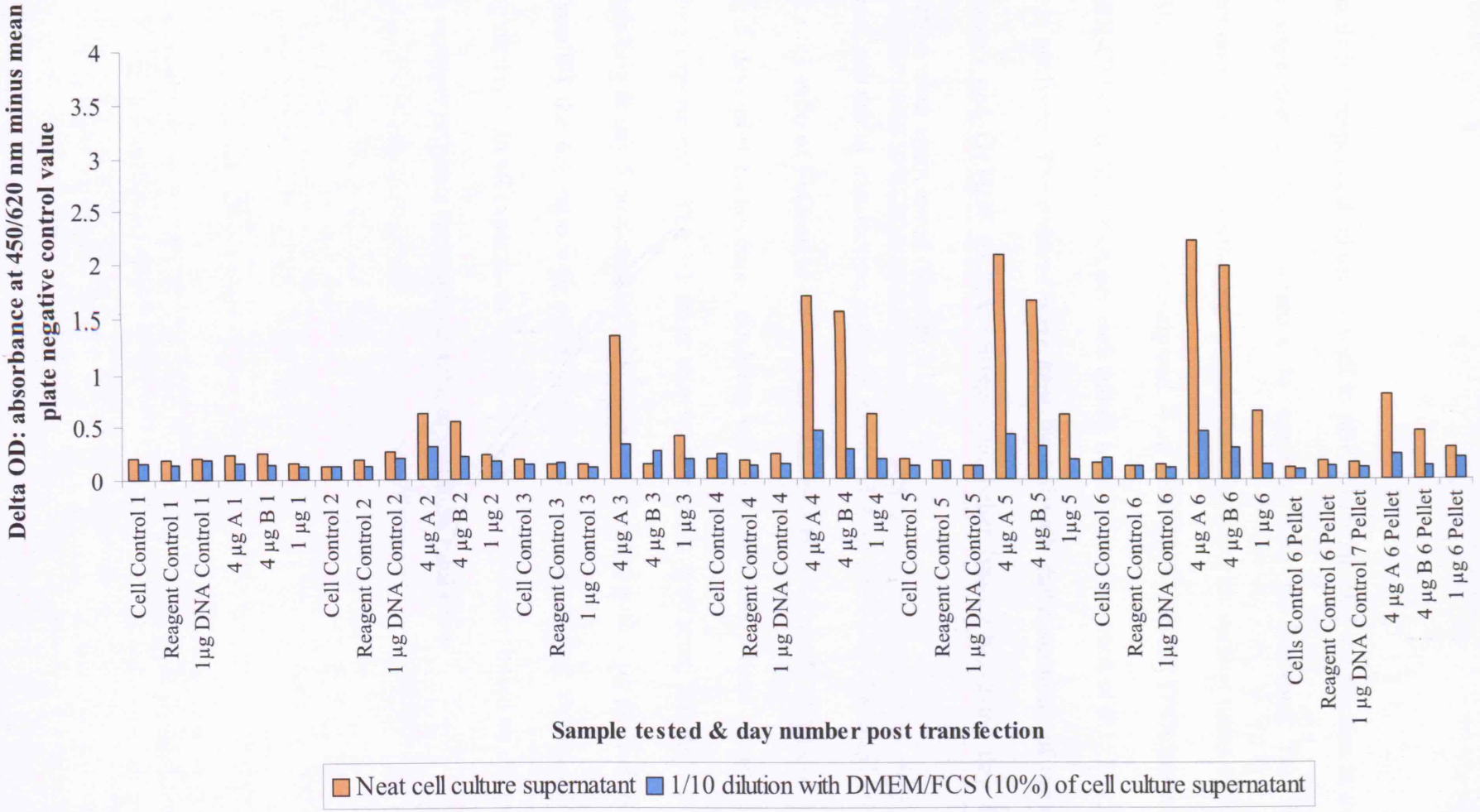


Figure 3.16 - Cos-7 transfection with wild type pBK-CMV[smallS]: results of Murex Ge34/36 HBsAg ELISA analysis of cell culture supernatant days 1-6 post transfection and cell pellet lysates day 6 post transfection



3.4.4: Optimisation of FuGene to pBK-CMV[smallS] DNA transfection ratios in CHO cells

CHO cells had responded relatively well to pBK-CMV[smallS] transfection in the time course experiments. In an attempt to improve their performance, the FuGene transfection process was further optimised. Transfection with various ratios of FuGene and pBK-CMV[smallS] were investigated. 4 μg pBK-CMV[smallS] DNA per well and 1 μg pBK-CMV[smallS] DNA per well mixed with FuGene at ratios of 6:1, 3:1, 3:2 and 1:1 were analysed. The cultures were kept for 6 d with daily sampling of the culture supernatants and Ge34/36 ELISA testing. Cell pellet lysates harvested day 6 post-transfection were again tested (figures 3.17-3.18).

The most successful transfection protocol involved 4 μg pBK-CMV[smallS] DNA per well in a 6:1 ratio of FuGene to DNA. This produced HBsAg levels of around 6 ng/ml HBsAg 5 days post transfection, doubling what had previously been achieved in all preceding experiments. The 3:1 ratio also worked well, producing around 3.5 ng/ml, again peaking at day 5 post-transfection. When transfecting with 1 μg per well of pBK-CMV[smallS] the 6:1 ratio was again most successful, producing around 2.5 ng/ml peaking on day 5. In all experiments there was again no evidence, based on comparative ELISA analyses of lysate and supernatants, of secretion inhibition.

Figure 3.17 - CHO transfection with varying ratios of FuGene to 1 µg DNA: results of Murex Ge34 HBsAg ELISA of cell culture supernatants days 1-6 post transfection and cell pellet lysates day 6 post transfection

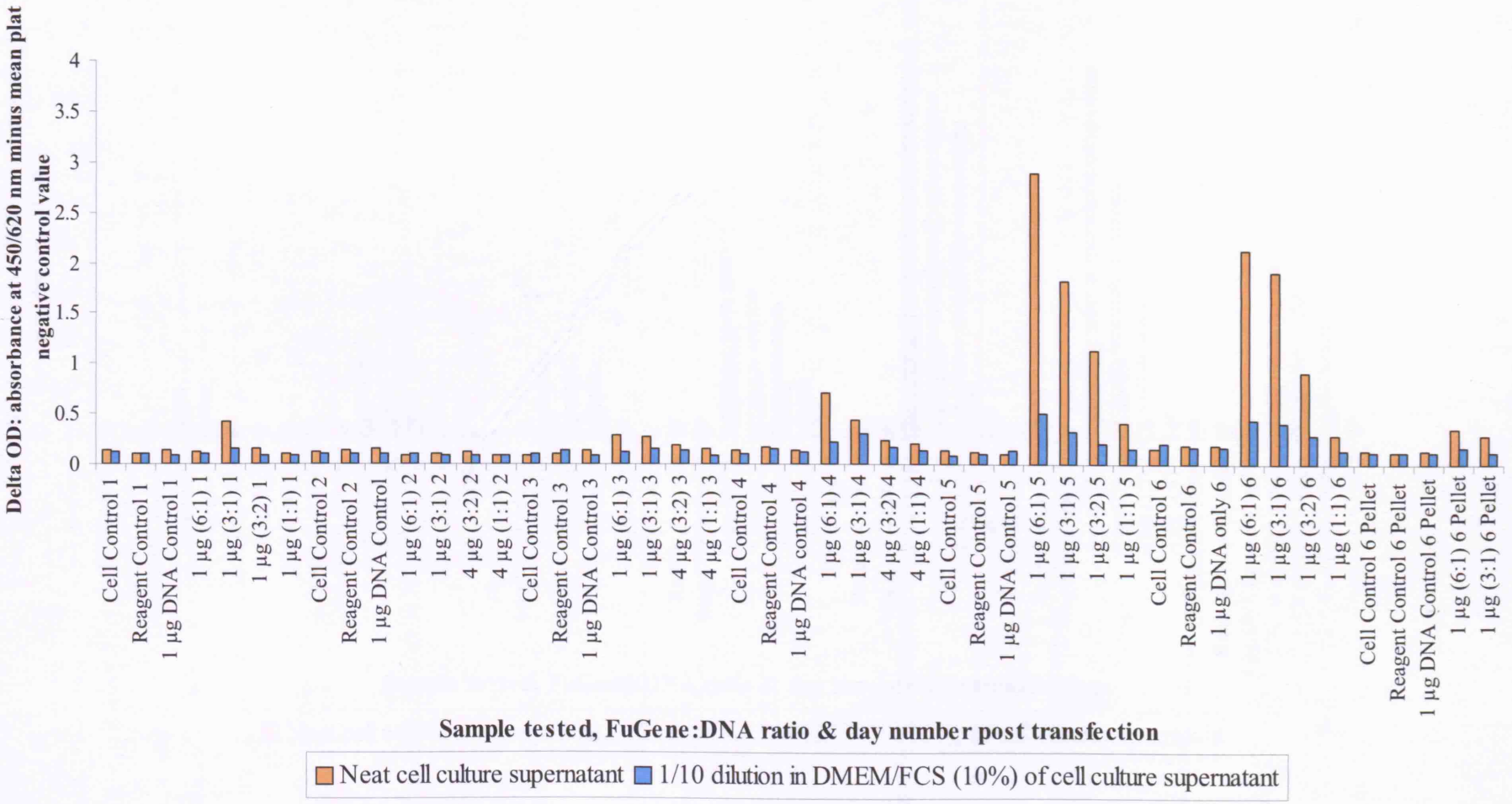
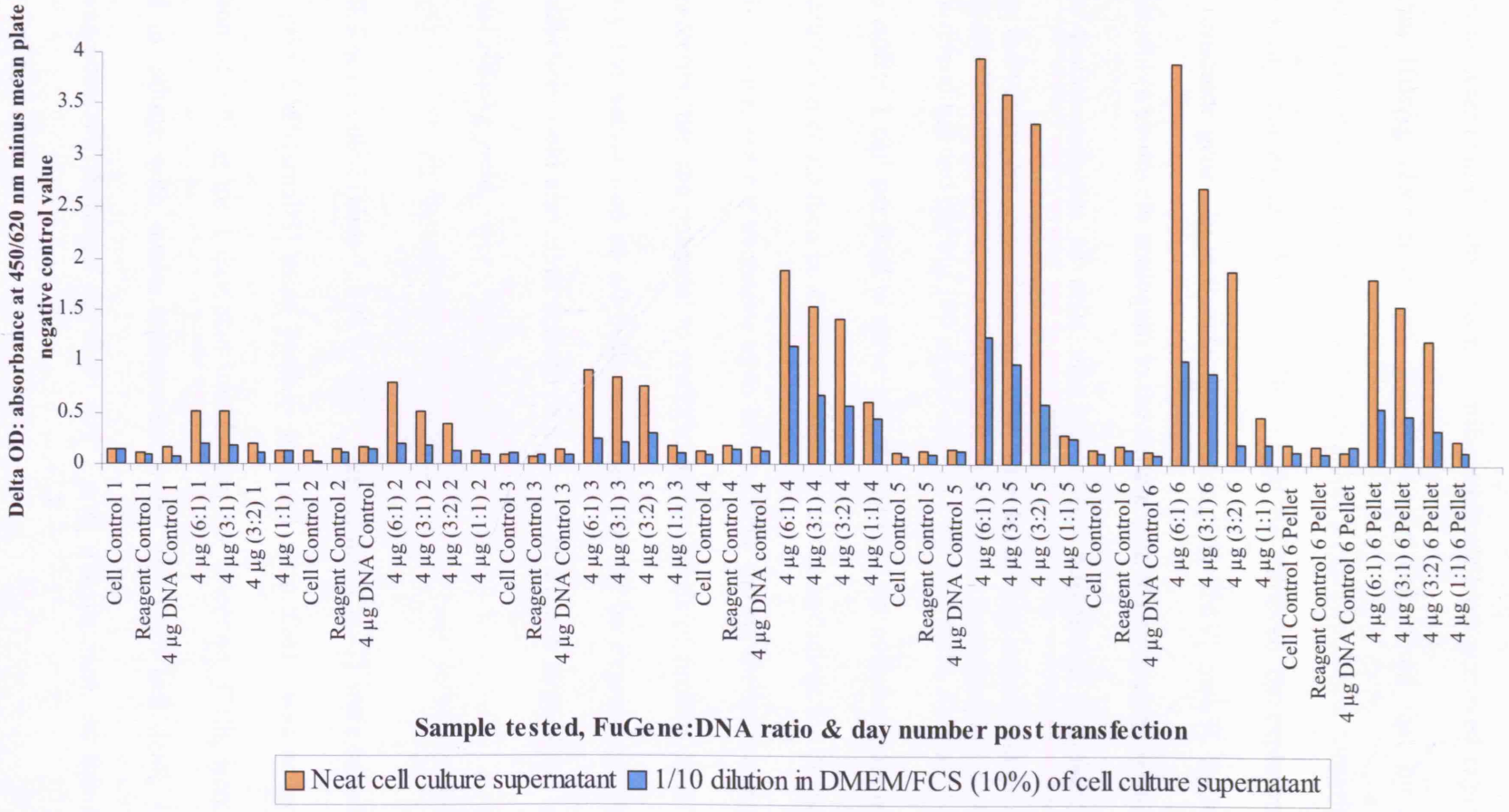


Figure 3.18 - CHO transfection with varying ratios of FuGene to 4 µg DNA: results of Murex Ge 34/36 HBsAg ELISA of cell culture supernatants days 1-6 post transfection and cell pellet lysates day 6 post transfection



3.4.5: Generation of stable cell lines transfected with pBK-CMV[smallS]

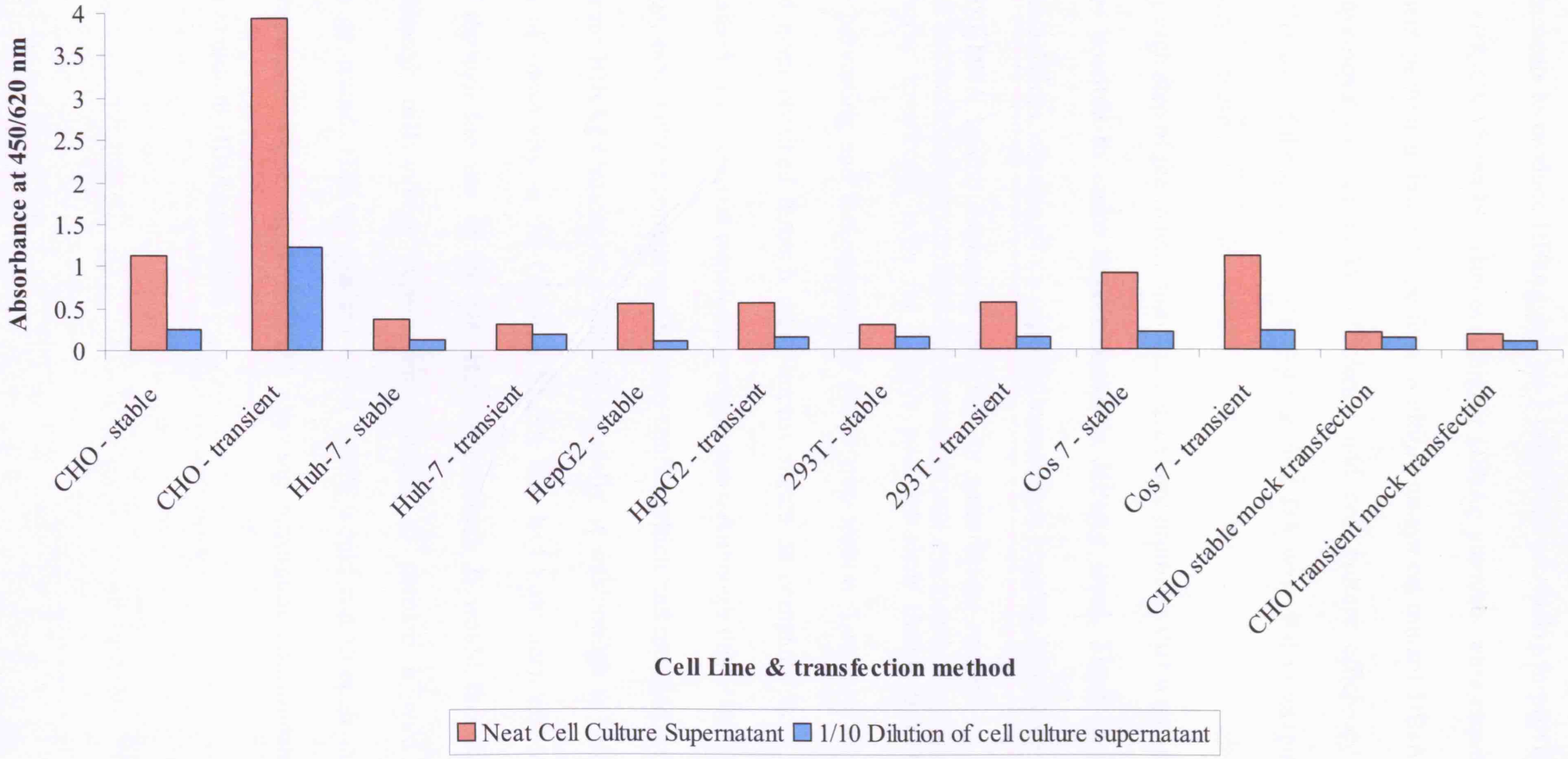
Although HBsAg production had been improved, levels of 6 ng/ml were still low in comparison to other studies which using similar methods have achieved expression of 30 ng/ml HBsAg (Jeantet *et. al.,* 2004). It was considered that by stable transfection of cell lines it might be possible to select a cell population comprised of a large majority that express HBsAg. This could be achieved by exploiting the neomycin resistance gene *neo* in transfection vector pBK-CMV[smallS]. By adding G418/Genetecin (a neomycin analogue) to the culture as a selective agent it might be possible to isolate colonies of cells that have been transfected and which are maintaining the vector. By re-seeding the resistant colonies at a concentration of 0.5 cell/100 µl of medium and plating 100 µl per well in a 96-well plate, it would then be possible to isolate 1 cell per well to serve as precursors of monoclonal populations. G418 concentration could then be kept at a lower level but sufficient for the cells to maintain the vector, but not excessive as to kill the cells during the selection stage. Stable transfection has the potential to result in higher levels of protein as all cells should carry the vector and so all cells in culture should be expressing HBsAg. Stable transfection could also allow cells to be cultured on a much larger scale and so increase total HBsAg yield.

All the cell lines studied (Huh-7, 293T, CHO, HepG2 and Cos-7) were transfected with 4 µg pBK-CMV[smallS] using FuGene in a 6:1 ratio. G418 was added at a concentration of 800 µg/ml 1 day post transfection for selection. Cells were then maintained in culture with media replacement every day until cell death in the majority was observed (between day 3 to day 7 post transfection). At this stage,

resistant colonies which remained were re-seeded at a G418 concentration of 300 μg/ml to generate successively larger cultures. Once a suitable culture size had been reached, the cell culture supernatant was harvested from cultures that had attained confluence for at least 4 days (allowing HBsAg levels to accumulate). The supernatant was then assayed in the Ge34/36 ELISA (figure 3.19)

CHO cells were again found to be the most productive, secreting 0.7 ng/ml of HBsAg in comparison to 0.5 ng/ml for Cos-7s, 0.1 ng/ml for Huh-7s, 0.25 ng/ml for HepG2s and 0.1 ng/ml for 293Ts. But these levels were low in comparison to those obtained from transient transfection. A possible explanation for their comparative poor performance following stable transfection was the selection and isolation of single resistant clones which were 'pseudo resistant'. Pseudo-resistant cells do not contain the vector and are often healthier as the uptake of foreign DNA can be damaging. When re-seeded inadvertently as a mixed population the healthier pseudo-transfected cells may have out-competed the truly transfected cells thus resulting in the low HBsAg levels observed. This problem may be overcome by more carefully calculating killing selection doses and maintenance doses of G418 specifically for the individual cell line or by isolating single true resistant cells with a pipette tip rather than by dilution. Nonetheless, given that transient transfection was far more convenient, stable transfection work was discontinued.

Figure 3.19 - Stable Transfection vs. transient transfection of various cell lines with wild type pBK-CMV[smallS]: results of Murex Ge34/36 HBsAg ELISA analysis



3.4.6: Cell culture optimisation overview

Initial experiments to produce HBsAg in Cos-7 cells were promising in proving the function of pBK-CMV[smallS]. However higher HBsAg amounts were required to have sufficient protein in order to perform multiple assays on mutant HBsAg. As such, improvements in transfection efficiency and cell culture efficiency were necessary. The use of the quantified HBsAg standard DA was vital in gauging the success of any of these optimisation efforts.

By assaying each step of the transfection and cell culture protocols that were initially used it was possible to make improvements in HBsAg level. These included, changing the cell line, changing to a superior transfection reagent, increasing pBK-CMV[smallS] DNA levels, increasing the ratio of transfection reagent to vector DNA, culturing transfected cells for 5 days post transient transfection before supernatant harvesting and the rejection of stable transfection. Levels of 6 ng/ml HBsAg had been obtained through these improvements as compared to 3 ng/ml HBsAg obtained in the earliest transfection experiment. Although this HBsAg level was not large, especially in comparison to other studies which had reported levels of up to 30 ng/ml HBsAg (Jeantet *et. al.,* 2004), critically, it was enough to reach the upper limit of sensitivity in the Ge34/36 ELISA that had now been empirically determined through the use of the DA HBsAg standard. It would therefore be possible, through cell culture supernatant dilution, to prepare a wild type, transfection generated, HBsAg standard curve which would run through the full range of absorbance values in the ELISAs allowing maximum discrimination in comparison to mutant HBsAg samples.

3.5: Optimisation of single monoclonal, multiple monoclonal and polyclonal capture ELISAs with wild type *in vitro* generated HBsAg

It had been demonstrated that transient transfection of CHO cells with pBK-CMV was a suitable method for generating wild type HBsAg in sufficient quantities for analysis with the Ge34/36 ELISA. Although HBsAg levels of 6 ng/ml were obtained that were enough to generate peak ELISA absorbances, it was desirable to increase ELISA sensitivity in order that large volumes of cell culture need not be generated The monoclonal antibodies (MAbs) P2D3 (Ijaz *et. al.,* 2003), D2H5 and H3F5 (Tedder *et. al.,* 1983) bind distinct regions of the 'a' determinant of HBsAg (residues 122-127, 131-142 and 142-147 respectively) and their use in ELISA format would allow the three epitopes to be probed for each HBsAg mutant of interest. In fact the combination of all three MAbs held in the solid phase of a sandwich ELISA with polyclonal anti-HBs-conjugate detection is the basis of the Ge34/36 assay. However unlike the Ge34/36 ELISA kit, conditions would have to be optimised for use of each with the three MAbs.

Mutant HBsAg was also to be assayed for reactivity with vaccine induced polyclonal anti-HBs. This assay though not generating specific epitope data, as with the use of the MAbs.

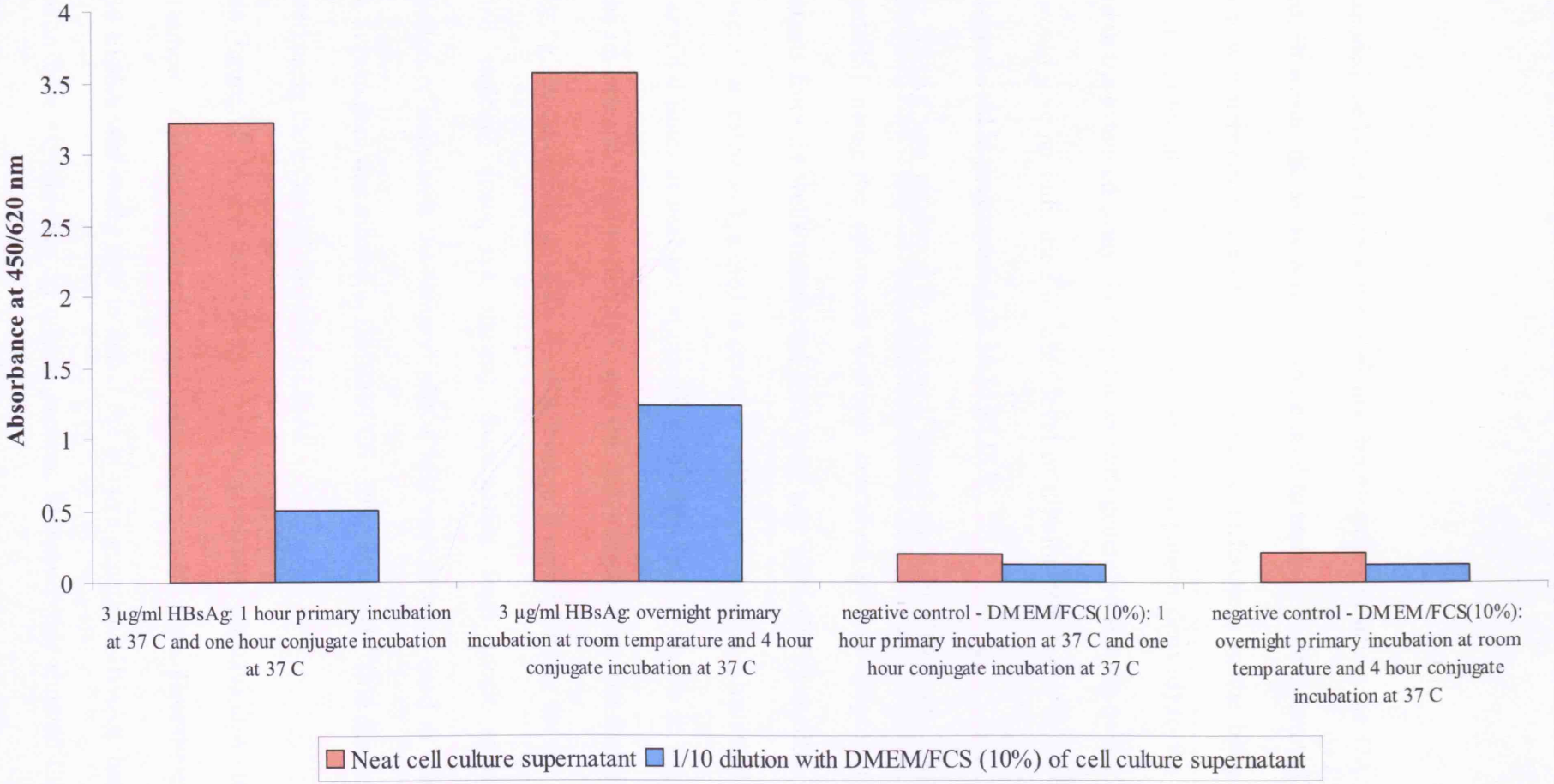
This chapter describes the changes made to the Ge34/36 ELISA to improve sensitivity as well as the optimisation of the MAb capture assays that were to be based on the related Ge34/36 ELISA. It also describes the isolation of polyclonal anti-HBs and the development of a sandwich ELISA using this as the capture component.

3.5.2: Increasing sensitivity of the Ge34/36 HBsAg ELISA

The simplest route to improving the sensitivity of an already established ELISA is to increase incubation times of the various steps. The Ge34/36 ELISA is a sandwich assay and so there exists potential to increase sensitivity at the sample incubation stage and also the conjugate incubation stage. The standard Ge34/36 ELISA protocol (as suggested by the manufacturer) involved a 1 h incubation with the sample at 37^0C with a 1 h conjugate incubation at 37^0C followed by incubation with TMB for 30 minutes at 37^0C. Using the DA HBsAg standard diluted to 3 ng/ml HBsAg incubation times were assayed in comparison to a protocol with an overnight (c. 16 h) room temperature (c.20^0C) sample incubation followed by a 4 h conjugate incubation at 37^0C and a standard 30 min TMB incubation.

It can be seen that the extended assay format did result in increased Ge34/36 sensitivity (figure 3.20) as the OD recorded for the neat extended time sample was slightly higher at 3.556 as apposed to 3.221 for the standard incubation length sample. Critically, negative control results increased by only a small amount. Moreover the result for the 1 in 10 dilution (0.3 ng /ml HBsAg)in the extended time format gave a much improved OD, rising from 0.495 to 1.233. This simple optimisation finally ensured that the HBsAg generated by transfecting CHO cells with pBK-CMV to a level of 6 ng/ml would be sufficient to conveniently culture and dilute down for use in the sensitised Ge34/36 ELISA

Figure 3.20 - Increasing length of incubation steps in Ge34/36 ELISA with DA HBsAg standard



3.5.3: Assaying *in vitro* generated HBsAg in the extended incubation Ge34/36 ELISA

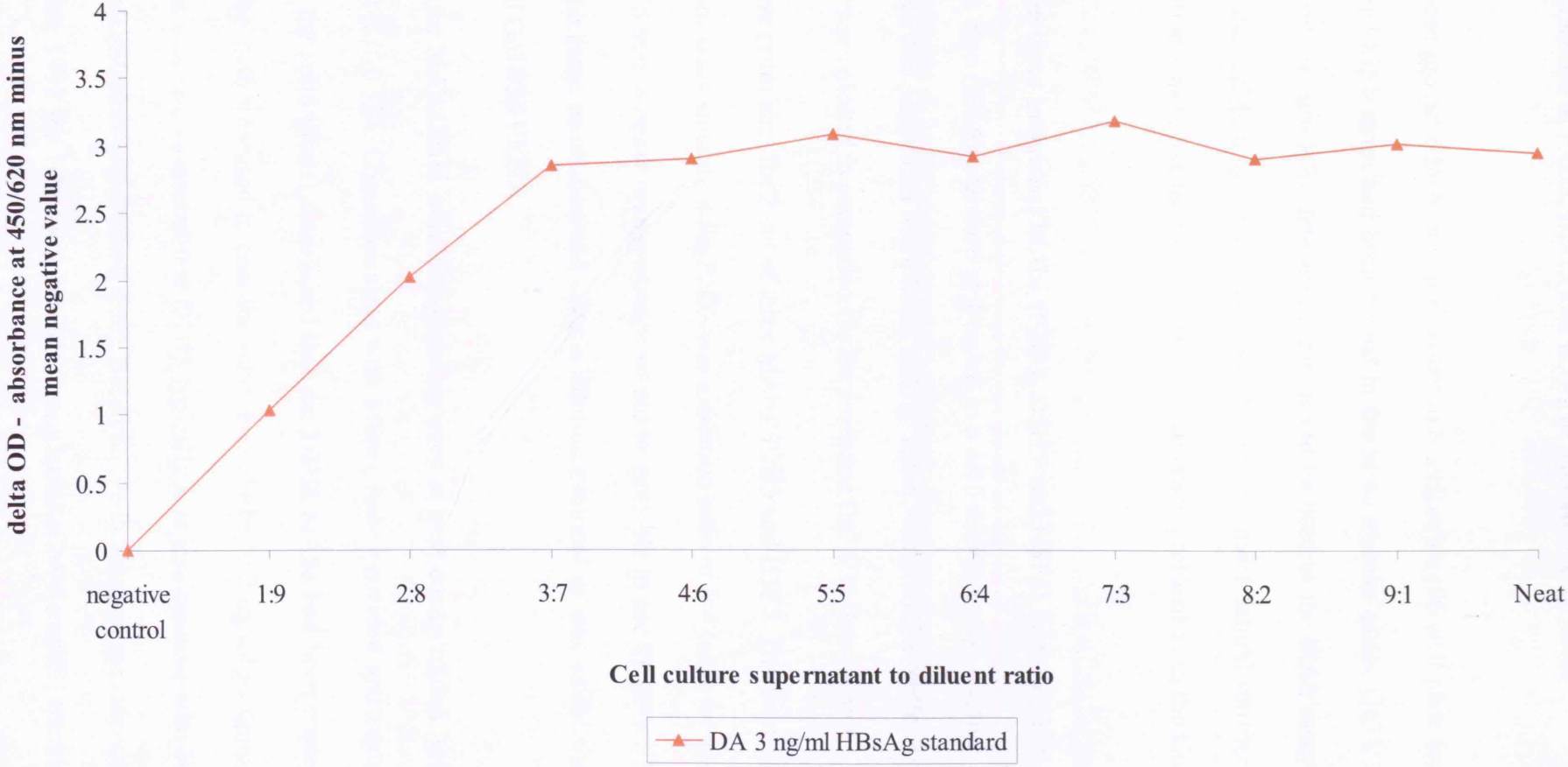
The extended Ge34/36 ELISA protocol had been optimised using the DA HBsAg standard. However, the assay was also to be used to assay *in vitro* generated HBsAg and so it was necessary to ensure there were no differences in the behaviour of protein of a different origin (*in vitro* as opposed to patient derived) to the HBsAg standard in the extended assay. Testing the *in vitro* generated HBsAg over a dilution series would give an indication of what level of dilution HBsAg of *in vitro* origin would be required in the extended Ge34/36 ELISA.

In vitro HBsAg was produced by transient transfection of CHO cells with pBK-CMV[smallS] using the optimised protocol described in this chapter. Clarified supernatants from 24 wells transfected with wild type pBK-CMV[smallS]in 6 well plates were harvested and pooled to create a wild type standard. This standard was then assayed 4 times at neat and at sample to dilutent rations shown in figure 3.21 using the un-extended Ge34/36 ELISA, and the data compared against the quantified DA HBsAg standard. This gave an HBsAg level of 5 ng/ml HBsAg for the *in vitro* generated standard (data not shown). Supernatant from mock transfections (transfection of cells with the 'empty' pBK-CMV vector) were used as a negative control. Supernatant was diluted in DMEM/FCS (10%) in a $1/10^{th}$ fold dilution series and tested using the extended Ge34/36 ELISA.

The data (figure 3.21) show that the *in vitro* HBsAg standard plateaus down to a ratio of 3:7 (sample : diluent) and that it is still detectable at a 1:9 ratio. Thus the extended Ge34/36 ELISA was easily able to detect the *in vitro* generated HBsAg that was to be used in later experiments on mutant protein. Moreover this showed that a the

dilution series from neat down to a 1 in 10 dilution was suitable for demonstrating the reactivity for *in vitro* generated HBsAg in this ELISA. Though wild type HBsAg saturated the ELISA until lower dilutions, there was sufficient range to assay samples that may be less reactive over the same dilution series.

Figure 3.21 - Mean results of in vitro generated (CHO transfection) wild type HBsAg in extended Ge34/36 ELISA.



3.5.4: Optimisation of P2D3, D2H5 and H3F5 MAb capture ELISAs

MAbs had been provided by Murex pre-coated in 8 well strips (96 well plate format). The individual MAb strips had been coated in the same manner as the Ge34/36 kit, also the polyclonal anti-HBs detection conjugate to be used in the MAb assays was the same as the Ge34/36 kit. Given these similarities the most natural starting point for optimisation was to use the same extended incubation protocol as in the Ge34/36 assay.

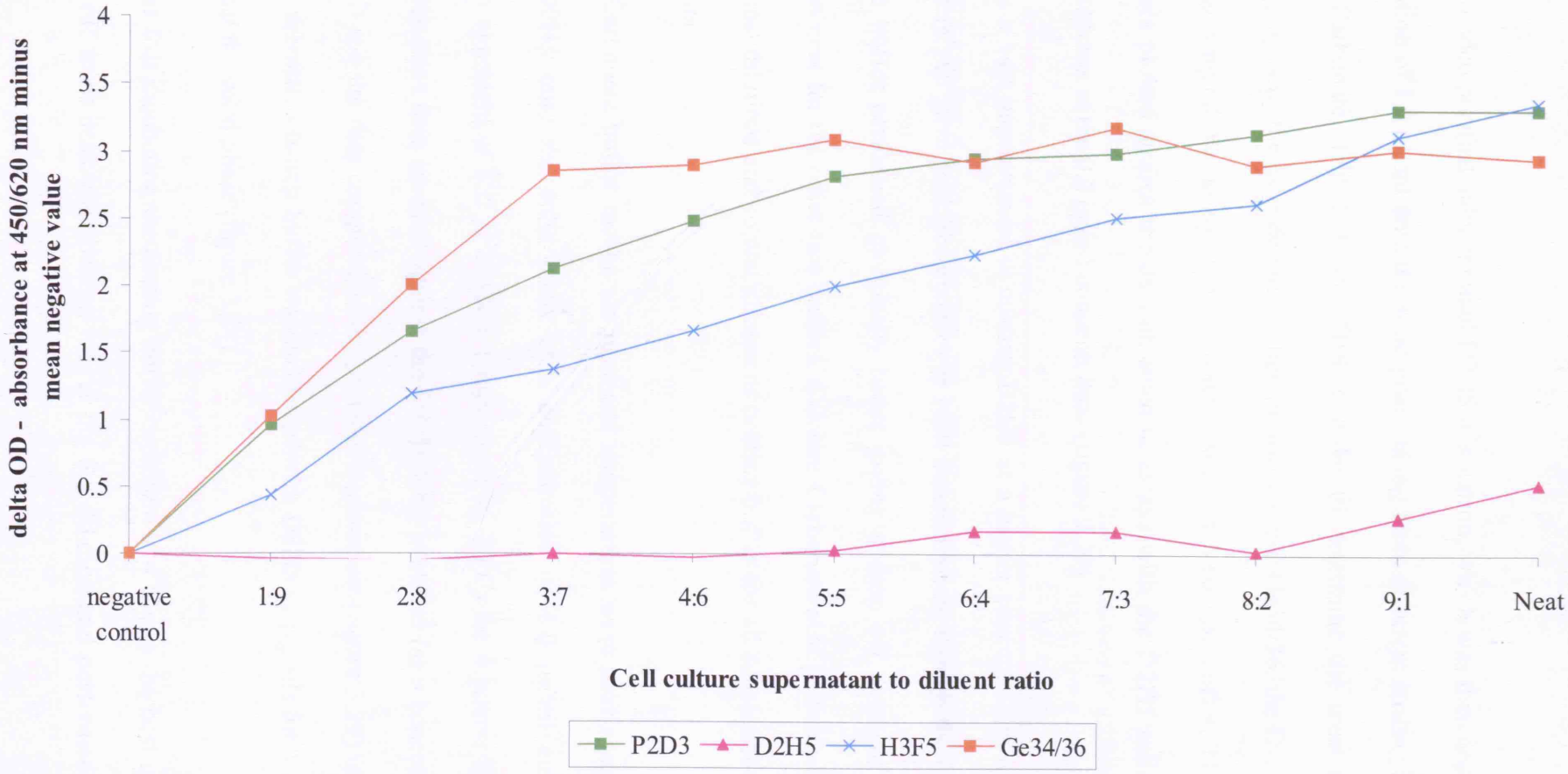
Therefore the three individual MAbs (P2D3, D2H5 and H3F5) were used to assay the DA wild type HBsAg standard at 3 ng/ml in a 1/10 dilution ratio. Testing of the dilution series was performed in triplicate and the mean results of the replicates from each testing were plotted in comparison to the extended Ge34/36 data (figure 3.22).

The data were promising for 2 out of three MAbs, P2D3 and H3F5. The dilution drop off point from assay saturation for P2D3 was a dilution ratio of 6:4 (sample: dilutent) and for H3F5 9:1. Without optimisation it would be possible to use these two MAbs in exactly the same incubation and sample dilution protocol as was established for the extended Ge34/36 ELISA.

The results for MAb D2H5 were disappointing even at neat concentration, giving a delta OD of just 0.524. Communication with Murex (who provided and coated the MAbs onto the solid phase) established that the D2H5 MAbs had been coated at a lower dilution than was used to coat the other two MAbs; 0.2 µg/ml as opposed to 1.0 µg/ml. It was also suspected that D2H5 typically was less reactive with HBsAg of *ad* serotype (Murex –unpublished data). Based on predicted serotype (according to Norder *et. al.,* 1992 the HBsAg generated through pBK-CMV[smallS] transfection

would be of subtype *adw2*. Therefore the first necessary optimisation experiment would be to assay HBsAg against D2H5 at 1.0 μg/ml concentration.

Figure 3.22 - Absorbance results of 'DA' standard HBsAg at 3 ng/ml in single monoclonal capture and multiple monoclonal (Ge34/36) capture ELISAs



3.5.5: Optimisation of D2H5 concentration and coating protocol

Murex provided purified, concentrated D2H5 in solution, which was then coated at a concentration of 1.0 μg/ml onto the solid phase using three different coating buffers; Calcium Carbonate, Tris-HCL and PBS in order to determine the most suitable choice. Then, using the extended incubation protocol as per Ge34/36, the DA HBsAg standard at 3 ng/ml was assayed in triplicate against each coating buffer. The mean results were plotted against the data obtained in assays with the D2H5 solid phase coated by Murex at the 0.2 μg/ml concentration (figure 3.29)

There was a vast improvement in coating D2H5 at a higher concentration using all three buffers. All gave peak absorbances at Neat concentration, though the Calcium Carbonate buffer performed marginally better giving a drop off point of 9:1 as opposed to neat for the other two buffers. Calcium Carbonate also performed better through most dilutions and so was chosen as coating buffer for all subsequent D2H5 experiments

Calcium Carbonate buffer incubation time and temperatures were next assayed by coating D2H5 onto the solid phase at a concentration of 1.0 μg/ml either by incubation overnight at 4^0C or at room temperature (c. 20^0C) for 4 hours. The two solid phases were then assayed against the DA HBsAg standard (at a concentration of 3 ng/ml) and the data compared to the previous experiment (figure 3.29) in which Calcium Carbonate coating buffer was incubated with D2H5 (1 μg/ml) for 1 hour at 37^0C to coat the solid phase (figure 3.24).

It was clear that incubating the coating buffer overnight at 4^0C was the best method. The drop off from peak absorbances was at the 6:4 dilution and performance was

superior at all lower dilutions. Coating buffer incubation at room temperature for 4 hours was the least effective method.

By changing D2H5 concentration and by the optimisation of D2H5 coating the D2H5 capture assay was now functional and used the same ELISA incubation conditions and would be effective over the same dilutions as the Ge34/36, P2D3 and H3F5 capture ELISAs.

Figure 3.23 - Comparision of two concentrations (0.2 µg/ml vs 1.0 µg/ml) of capture Mab D2H5 in an ELISA with quantified DA HBsAg standards using different Mab coating buffers

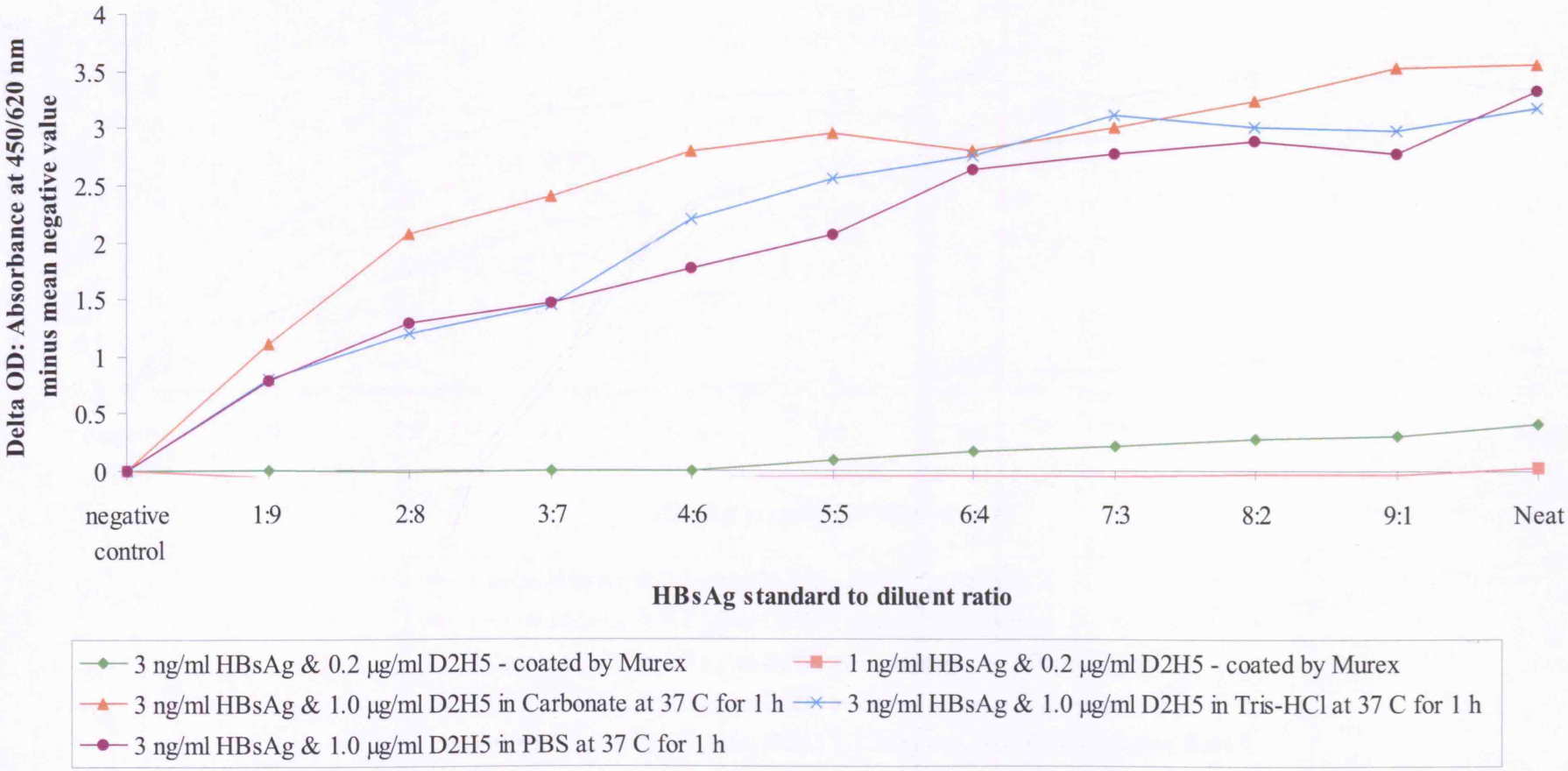
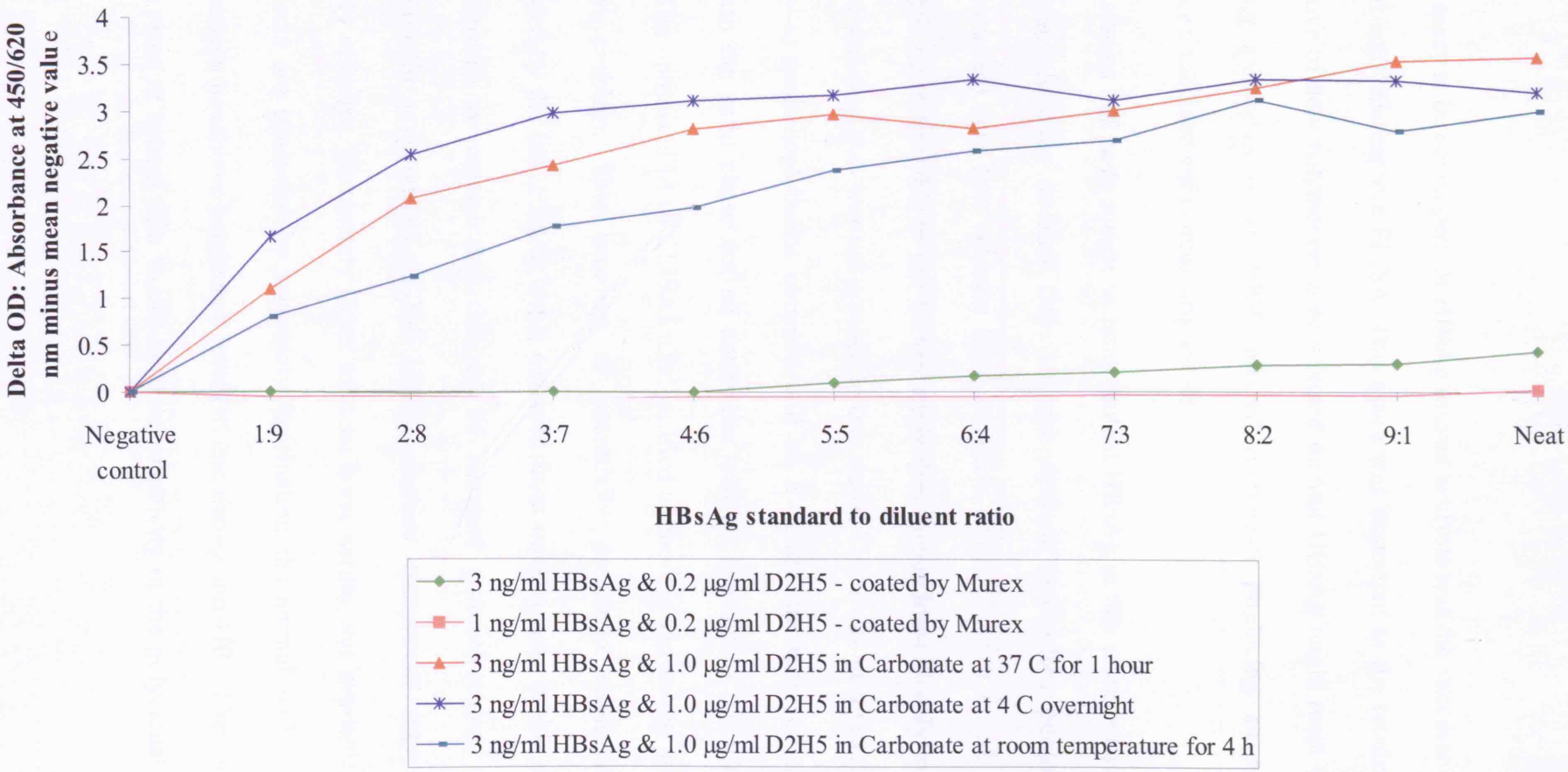


Figure 3.24 - Comparision of Mab coating protocol (times and temperatures) using Calcium Carbonate coating buffer in D2H5 capture ELISA with quantified DA HBsAg standard.



3.5.6: Development of polyclonal anti-HBs capture ELISA

The final assay to be developed for HBsAg mutant analysis was the vaccinee derived polyclonal anti-HBs capture ELISA. This assay was important as the results would be indicative of how polymerase gene selected mutant HBsAg might react with the neutralising antibodies of an HBV infected individual producing an anti-HBs response or a vaccinee with circulating anti-HBs.

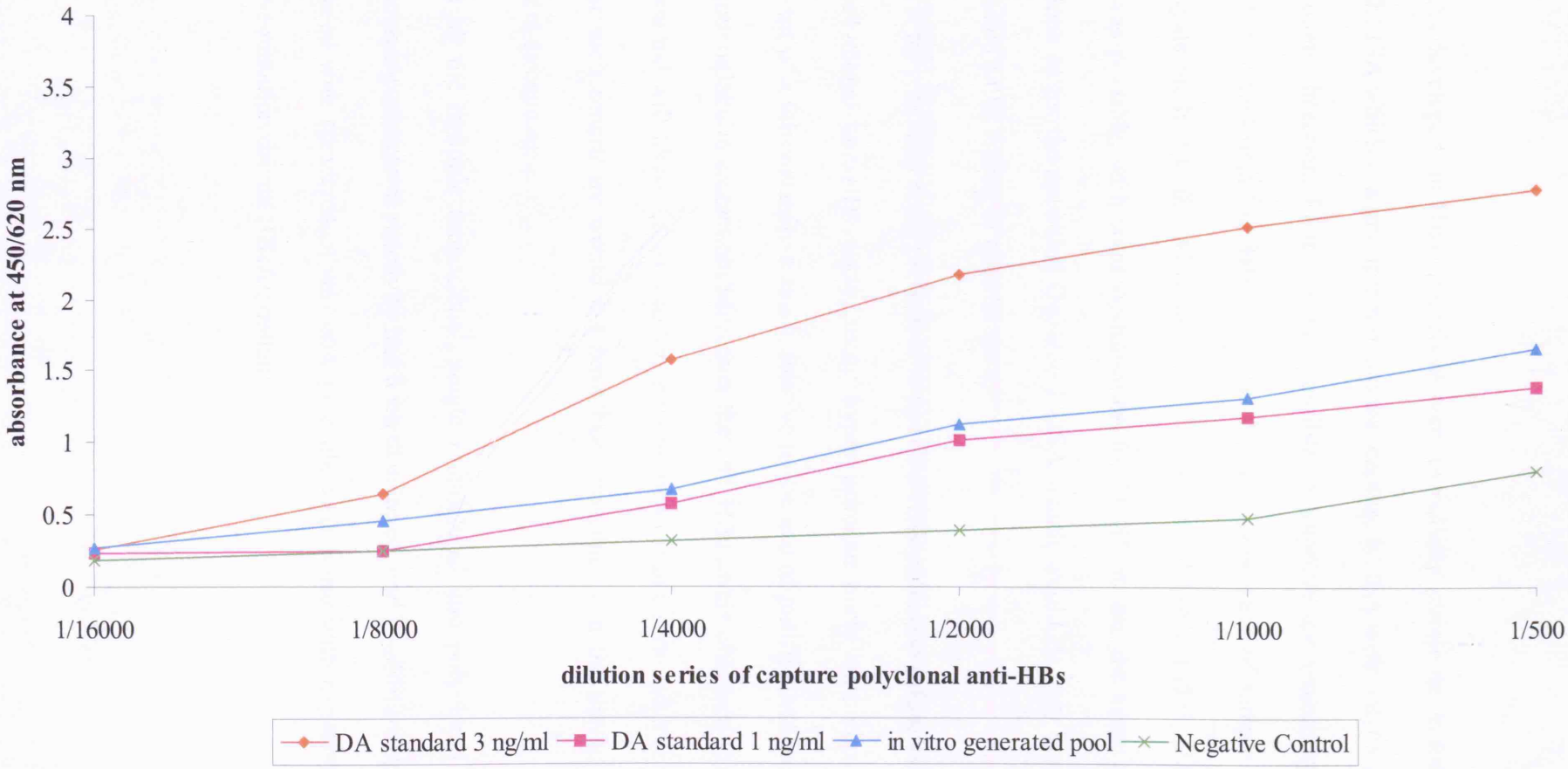
Previous studies that have sought to assay mutant HBsAg in this manner have often used immunoglobulins isolated from vacinee derived (and often pooled from multiple sources) sera that contains high anti-HBs levels (Toressi *et. al.,* 2002). Though it is most desirable to use human antibodies, hence most closely mirroring the *in vivo* situation, this was not pursued in this study. There was decreased ELISA sensitivity as immunoglobulins obtained will be a 'crude' mixture and so when coated onto the solid phase not all antibodies will be specific to the protein of interest. The optimised MAb ELISAs used purified antibodies and so did not suffer from this problem. This problem of potentially decreased sensitivity was compounded by the low HBsAg levels obtained from transfections in this study. It was reasonable to assume that the use of standard patient/vaccinee derived immunoglobulin preparations as the ELISA capture component may not be particularly effective. Fortunately hyper immune horse serum was available. Hyper immune sera are generated by repeatedly vaccinating the animal with the HBV vaccine antigen to achieve heightened levels of circulating anti-HBs. The use of this serum in place of human sera would improve sensitivity in the polyclonal capture assay.

Immunoglobulins (and therefore anti-HBs) were isolated from the horse serum by ammonium sulphate precipitation (salting out) at the isoelectric point (pI) for horse antibodies (the pH at which the protein has a net charge of zero and is thus minimally soluble), followed by dialysis to replace high salt buffer with low salt buffer.

This immunoglobulin preparation (containing polyclonal anti-HBs) was then used to coat the solid phase in a capture ELISA at dilutions ranging from 1/500 to 1/16,000. These were then used to assay the DA HBsAg standard at 3 ng/ml and 1 ng/ml as well as *in vitro* generated HBsAg (figure 3.25).

A dilution of 1/1000 was optimal, giving the greatest difference in absorbance between the negative control and the positive control samples. At the lower 1/500 dilution the negative control result would give too high a 'background' reactivity in the assay. Similarly at higher immunoglobulin dilutions the peak absorbances would be too low. In this instance the results from the *in vitro* generated HBsAg are almost as low as the 1 ng/ml DA HBsAg standard, though the *in vitro* HBsAg used in this experiment originated from the transfection of only one well and may be due to a single less effective transfection. Such problems could be avoided in the future by pooling supernatants from transfected wells and by performing repeat transfections to be assayed also.

Figure 3.25 - Comparison of polyclonal anti-HBs capture antibody diltutions against DA HBsAg standard and in vitro generated pooled HBsAg



3.5.7: ELISA optimisation overview

All ELISAs developed for HBsAg analysis were essentially variations on the Murex Ge34/36 ELISA which is a multiple monoclonal capture ELISA with polyclonal anti-HBs-conjugate detection. Firstly it was possible to improve the sensitivity of the assay, to better deal with low HBsAg levels, by increasing time of sample binding and conjugate binding steps. When assaying the individual MAbs P2D3, D2H5 and H3F5 it was possible, with some optimisation for D2H5, to use the same protocol and dilutions as for the extended Ge34/36 ELISA which would be very convenient for large scale batch testing of transfectants.

Although it may initially seem preferable to use anti-HBs of human origin as capture for the polyclonal anti-HBs assay, using hyper immune horse sera allowed the development of a fairly sensitive assay, relative to the use of purified MAbs, from a crude immunoglobulin extraction. Moreover the anti-HBs levels observed in a hyper immune animal will likely far exceed those found in vaccinee sera and it is entirely likely that such sensitivity would not have been possible with the use of human vaccine sera as capture.

Now that all the multiple monoclonal, single monoclonal and polyclonal capture ELISAs to assay changes in reactivity had been developed, and in addition optimised and validated with standards, it was now possible to proceed with experiments on polymerase mutation driven HBsAg mutants.

CHAPTER 4

HBsAg BINDING ANALYSES USING P2D3, D2H5, H3F5, GE34/36 AND HORSE ANTI-HBS OF SUPERNATES AND LYSATES FROM CHO CELLS TRANSFECTED WITH HBV S GENE MUTANT pBK-CMV[smallS] CONSTRUCTS

4.1: Introduction

The purpose of this study was to investigate changes in the binding of HBsAg in supernates of cells transfected with constructs bearing mutant HBV *S* genes to MAbs that specifically bind distinct epitopes of the HBsAg "a" determinant, and to polyclonal anti-HBs. The mutant HBsAg proteins for study were chosen because they represent mutations of the small S gene that may be selected when antiviral resistance-associated mutations develop in the overlapping *pol* gene.

The previous chapters describe how the transfection vectors carrying the mutant small *S* genes were constructed, transfections and protein production from cell cultures optimised, and capture ELISAs validated. CHO cells were transfected with each of the mutant and wild type plasmids (4 wells of a 6 well plate per transfection). Upon harvesting, the supernate was clarified and pooled. Cell pellets were also collected following which they were subjected to non-denaturing lysis. Supernates and lysates were then tested for the presence of HBsAg. All transfections were performed in triplicate and the resulting supernate pools individually assayed in duplicate. Results were represented as means of the duplicate testing of the three separate transfection pools (i.e. 4/6 wells of a 6 well plate x 3 transfections assayed twice). Such replication ensured that the effect of variation due to differential transfection efficiency per well was minimised.

A series of 10 fine dilutions of the supernates were assayed in an ELISA across a single 10-fold range using P2D3, H3F5 and D2H5 as solid phase capturing antibodies. Each dilution point was assayed in duplicate on each ELISA plate and there were 10 dilution points per HBsAg, so a further degree of precision was ensured. Results were validated by assaying

replicates of the DA HBsAg standard, a native *adw* HBsAg internal control as used at the UCLH Dept. of Virology and defined in potency by comparison against the WHO international HBsAg control. The DA standard was assayed at concentrations of 3 ng/ml HBsAg and 0.3 ng/ml HBsAg on each MAb ELISA plate.

4.1.2: Characteristics of the solid phase monoclonal antibodies

P2D3

The murine MAb P2D3 was raised against native HBsAg, derived from renal transplant patients, which harboured mutations in the second loop of the "a" determinant. These mutations rendered HBsAg undetectable by certain monoclonal-based assays, but detectable via polyclonal based assays. Through cross competition assays, the MAb was demonstrated to be reactive against wild type HBsAg and the second loop mutant HBsAg. The precise epitope for P2D3 was defined using ELISAs employing synthetic short (10-14 mer) oligo-peptides and was found to be in surface residues s121-129 (Ijaz *et al.*, 2003). In particular, the antigenicity of the oligo-peptides was found to be sensitive to the integrity of a TTP amino acid motif lying between s125-127 in the first loop. The P2D3 epitope was defined as linear as the MAb proved to be strongly reactive in Western Blot assays against denatured HBsAg. The MAb was assayed against a panel of 50 diverse sera from patients infected with HBV and the data contrasted to their DNA sequence. Only 5/50 samples demonstrated loss of reactivity with P2D3 and all were shown to contain a genotype defined variation within the TTP motif. Otherwise, the MAb was shown to be able to detect HBsAg despite large perturbations of the second loop epitope.

The recognition of a linear epitope was of crucial importance in this study as P2D3 effectively acts as a reporter for the presence of HBsAg in supernate and in lysates (as a component of the Ge34/36 assay). If the TTP motif is carried by the genetic backbone then HBsAg should be detected despite any HBsAg conformation. All transfected plasmids in this study bore a TTP motif. Hence a transfection supernate that did not react to P2D3 or only at a low level either results from inefficient transfection or transfection of a protein that for conformational or other reasons cannot be expressed. The P2D3 epitope is represented in figure 4.1 below.

H3F5 and D2H5

The monoclonal antibodies H3F5 and D2H5 were raised against a blend of *ad/ay* HBsAg and selected to recognise all 10 members of the Anne-Marie Courouce Paris panel of genotypes (Tedder *et al.*, 1983). The methodology for defining epitopes for MAbs H3F5 and D2H5 was slightly different, as longer (20-27 mer) synthetic peptides spanning the putative HBsAg 'a' determinant epitopes had been employed in dot blot assays using a "sliding" technique to progressively probe along the molecule (personal communication: Dr Julian Duncan, Murex Biotech Ltd, Dartford, Kent). It was initially demonstrated that D2H5 showed greatest reactivity with a peptide spanning s135-155 whilst H3F5 reacted best, but weakly with a peptide spanning s110-137. These two MAbs were further characterised against patient-derived HBsAg panels that bore known point mutations in distinct codons across the "a" determinant. H3F5 was shown to be unable to recognise mutations in the region of s120-135. Further, H3F5 reacted strongly with *ay* HBsAg but weakly with *ad* and was affected by changes around residue s122. D2H5 does not recognise antigens mutated in the region in which it reacts, i.e., s140-145.

In this way, the epitopes were mapped to regions between s131-142 for H3F5, and s142-147 for D2H5. They were demonstrated to be cross-competition related, but recognising distinct epitopes. H3F5 and D2H5 are variously sensitive to mutations of the second loop of the "a"

determinant and do not function in Western blot assays and so are considered to recognise conformational epitopes. The epitopes are represented in figure 4.1 below.

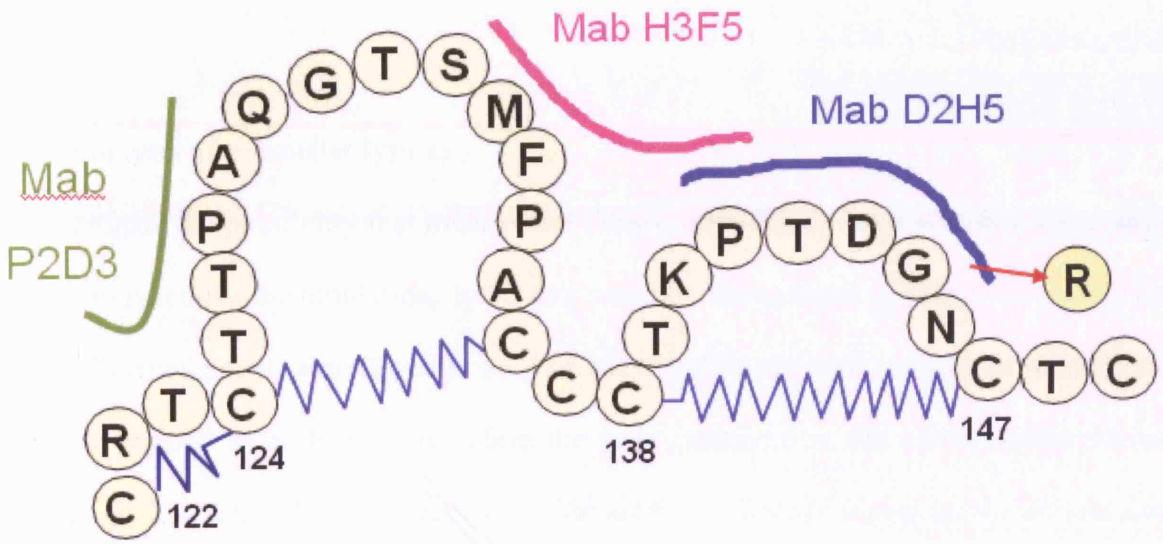


Figure 4.1 – Monoclonal antibody (MAb) binding epitopes on the "a" determinant of HBsAg. P2D3 binds surface residues s121-129, H3F5 s131-142 and D2H5 s142-147. The sG145R substitution mutation is also indicated. (Reproduced with permission, Dr Peng Yin, Abbott-Murex).

Multiple Monoclonal and polyclonal antibody assays

One-tenth-fold dilutions of the supernates were also assayed against the Ge34/36 solid phase used as a commercial micro plate reagent by Murex Diagnostics in the Ge34/36 HBsAg ELISA. A mixture of MAbs P2D3, H3F5 and D2H5 comprised the capture component of Ge34/36. As per the MAb capture assays, Ge34/36 supernates were assayed against horse polyclonal anti-HBs, isolated from a multiply-immunised hyper-immune animal. This reagent was routinely used by the NBS (National Blood Service) during the 1970's as a solid-phase antibody in a screening assay for blood donors.

4.1.3: Analysis of cell pellet lysates

To investigate the possibility that transfectant HBsAg may fail to secrete or that there may be HBsAg intracellular accumulation, lysed cell pellets were assayed undiluted in the Ge34/36 ELISA. The cell pellets were lysed in 1 ml of lysate buffer per well, in contrast to the 3 ml of supernate applied to each well; therefore the lysis represents a three-fold concentration of HBsAg. Binding ratios for supernates were divided by the lysate binding ratio to obtain a ratio which was used to infer the possibility of HBsAg secretion deficiency as previously described by Jeantet and colleagues (Jeantet *et al.*, 2004).

4.1.4: Analysis of data obtained from supernate capture assays

For all recombinant HBsAg dilution series and for each separate capture ELISA, the binding ratio (BR) was calculated. BR is defined as sample ELISA absorbance divided by the mean negative control [mock transfection] ELISA absorbance. The standard deviations (SDs) for all BRs from each dilution point for each mutant were also calculated. Dilution series curves

derived from mean BRs and standard deviations from each capture assay were then plotted (an example is shown in figure 4.2, below).

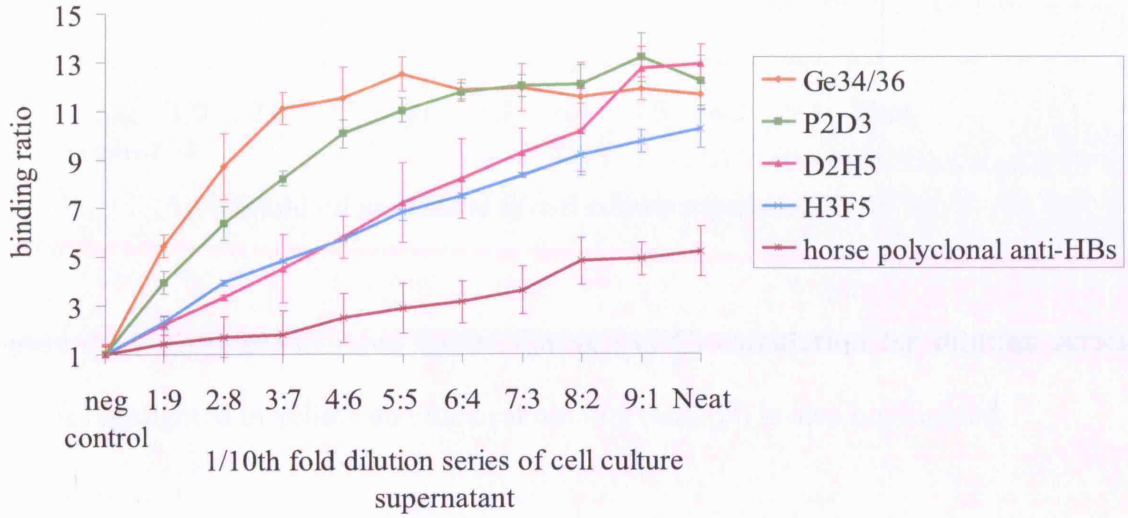


Figure 4.2 – Example of data; mean Binding Ratios (BR) of wild type HBsAg in a ten-fold dilution series.

The Area Under Curve (AUC) was then measured for each BR dilution curve for each capture assay (MAbs P2D3, H3F5, D2H5 as well as Ge34/36 and the horse hyperimmune serum). SD for each AUC measurement was derived from the HBsAg dilution series binding ratios. An example of AUC measurement can be seen below in figure 4.3.

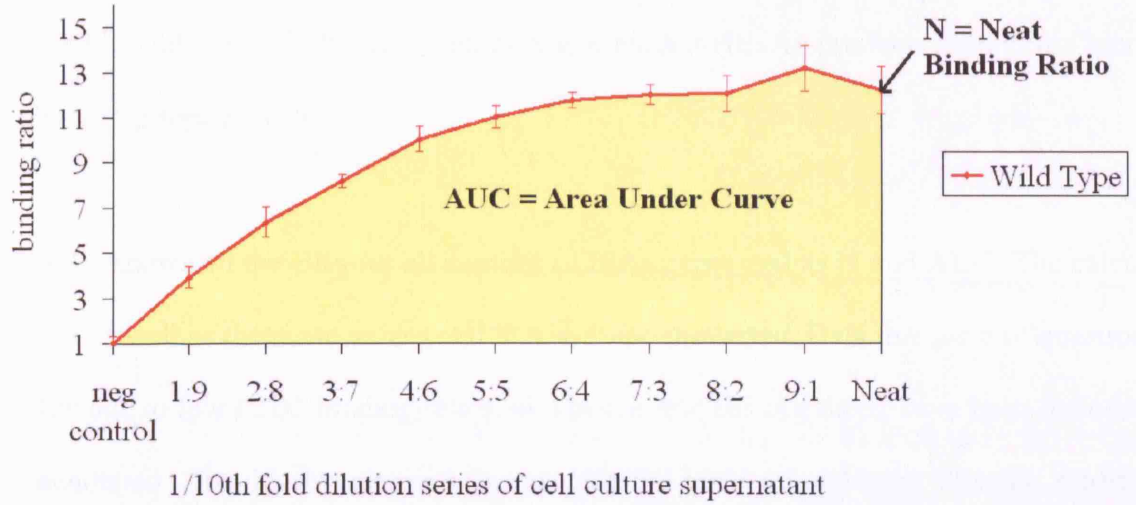


Figure 4.3 – Example of Area Under Curve (AUC) calculation for dilution series. The AUC is highlighted in yellow and the neat binding ratio (N) is also highlighted.

The AUC value was useful to define each epitope, as the dilution series is essentially a dose response curve which measures the potency of the transfectant for each capture antibody; thus AUC is effectively a measure of epitope potency when contrasted to wild type. The utility of this approach can be extended by considering relative epitope potency against a particular HBsAg binding to each of the three monoclonal antibodies used in solid phase assays. By combining the AUCs of an antigen for P2D3, H3F5 and D2H5 as a measure of total "HBsAg antigenicity" and then expressing the relative contribution of the individual solid phase reactions as a percentage ratio for each HBsAg, the "epitope balance" for each HBsAg could be calculated. This means of calculation allows inferences about epitope expression for each HBsAg to be made, regardless of supernate HBsAg titre, as the data are considered only as a relative ratio, with each MAb acting as the other's control. As an exercise, the wild type AUC

epitope balance was also calculated for pairs of MAbs (i.e. H3F5 and D2H5; P2D3 and H3F5; and P2D3 and D2H5) by excluding the third solid phase reaction producing a derived epitope density ratio for three different pairs; this derivatised ratio allowed meaningful contrasts to be made with wild type HBsAg in instances where mutant HBsAg produced would not bind to a MAb (an "epitope miss").

Table 4.1 shows all the BRs for all capture ELISAs expressed as N and AUC. The calculated cut-offs as well as the mean values and SDs are also presented. Data that were of questionable validity due to low P2D3 binding ratios, and hence low HBsAg titres, have been included but are annotated .The MAb epitope balance data for each recombinant HBsAg studied are presented in table 4.1.

		CAPTURE ANTIBODIES									
		P2D3 (121-129)		H3F5 (131-142)		D2H5 (142-147)		Ge34/36 (P2D3 +D2H5 + H3F5)		horse polyclonal anti-HBs	
Mutant	Antiviral	Binding Ratio in Neat Supernatant (N) +/- SD and Area Under Dilution Series BR Curve (AUC) +/- SD									
		N	AUC	N	AUC	N	AUC	N	AUC	N	AUC
Wild Type	~	12.2 +/- 1.2	85.1 +/- 3.5	10.3 +/- 0.8	53.9 +/- 3.7	12.9 +/- 0.8	60.3 +/- 3.7	11.6 +/- 0.7	92.7+/- 10.3	5.2 +/- 1.0	19.8 +/- 1.4
+ Cut-off	~	1.6	8.2	1.9	4.5	2.7	2.9	2.9	14.0	2.1	4.6
rtT128N/sP120T	Lamivudine	12.7 +/- 0.8	95.5 +/- 3.6	1.2 +/- 0.1	0.1 +/- 0.1	13.9 +/- 1.4	97.7 +/- 4.2	12.9 +/- 1.6	91.7 +/ 3.1	5.4 +/- 1.2	17.3 +/- 1.5
rtT128N/sP120T + rtM204V/sI195M	Lamivudine	12.3 +/- 0.4	76.5 +/- 3.5	0.9 +/- 0.2	-2.1 +/- 0.1	10.9 +/- 0.6	51.4 +/- 3.3	11.3 +/- 0.8	75.9 +/- 3.0	3.5 +/- 0.7	10.5 +/- 0.8
rtSilent/sD144E	Lamivudine	11.2 +/-0.5	85.7 +/- 3.0	9.4 +/- 0.1	53.8 +/- 2.9	10.3 +/- 0.4	54.5 +/- 2.6	11.5 +/- 0.8	95.2 +/- 3.0	5.3 +/- 0.5	16.2 +/- 1.4
rtSilent/sD144E + rtM204V/sI195M	Lamivudine	14.6 +/- 0.2	92.2 +/- 3.8	10.6 +/- 1.8	58.0 +/- 3.8	1.8 +/- 0.2	-0.4 +/- 0.4	15.0 +/- 1.2	95.0 +/- 3.9	1.6 +/- 0.9	4.4 +/- 0.3
rtR153Q/sG145R	Lamivudine	11.3 +/- 0.1	68.5 +/- 3.4	3.9 +/- 1.5	14.8 +/- 0.9	1.0 +/- 0.5	-1.3 +/- 0.1	10.7 +/- 0.5	59.0 +/- 3.1	1.0 +/- 0.5	0.1 +/- 0.1
rtR153Q/sG145R + rtM204V/sI195M	Lamivudine	12.4 +/- 2.1	67.5 +/- 3.8	8.6 +/- 0.1	35.4 +/- 2.4	1.1 +/- 0.4	-0.3 +/- 0.1	11.9 +/- 0.4	77.6 +/- 3.4	5.4 +/- 0.4	16.4 +/- 1.3
rtR153K/sD144EsG145R	Lamivudine	4.8 +/- 1.3	8.2 +/- 0.9	1.5 +/- 0.1	2.6 +/- 0.2	1.0 +/- 0.3	-2.0 +/- 0.1	3.3 +/- 0.4	14.1 +/- 0.9	1.5 +/- 0.4	1.0 +/- 0.3
rtR153K/sD144EsG145R + rtM204V/sI195M	Lamivudine	12.0 +/- 0.8	67.9 +/- 3.7	1.4 +/- 0.1	1.9 +/- 0.2	0.9 +/- 0.1	-1.1 - 0.1	12.1 +/- 0.5	64.2 +/- 3.4	1.4 +/- 1.0	3.5 +/- 0.2
rtF166L/sF158Y	Lamivudine	11.2 +/- 1.0	72.1 +/- 3.6	1.7 +/- 0.3	2.6 +/- 0.4	2.3 +/- 0.8	4.9 +/- 3.6	9.9 +/- 0.4	78.6 +/- 3.3	1.6 +/- 0.7	0.9 +/- 0.3
rtF166L/sF158Y + rtM204V/sI195M	Lamivudine	8.9 +/- 0.2	40.8 +/- 1.8	4.1 +/- 0.6	17.2 +/- 0.6	4.1 +/- 1.0	13.4 +/- 0.8	9.5 +/- 1.4	31.5 +/- 2.2	2.0 +/- 0.9	2.3 +/- 0.3
rtI169T/sF161L	Entecavir	11.2 +/- 0.5	74.0 +/- 2.8	10.6 +/- 0.9	53.4 +/- 3.3	2.9 +/- 1.2	5.3 +/- 0.5	10.3 +/- 1.2	77.5 +/- 2.3	3.6 +/- 1.2	11.1 +/- 0.7
rtV173L/sE164D	Lamivudine	12.5 +/- 1.1	77.0 +/- 3.6	8.1 +/- 0.6	40.2 +/- 2.2	12.0 +/- 1.3	50.2 +/- 3.1	11.4 +/- 0.5	82.2 +/- 3.3	4.4 +/- 1.0	14.0 +/- 1.3
rtV173L/sE164D + rtM204V/sI195M	Lamivudine	12.3 +/- 0.3	63.0 +/- 3.8	8.5 +/- 0.8	34.8 +/- 2.4	1.0 +/- 0.2	-1.2 +/- 0.1	12.9 +/- 1.2	77.9 +/- 3.6	1.5 +/- 1.0	1.4 +/- 0.2
rtL180M/sSilent	Lamivudine	12.7 +/- 0.7	85.0 +/- 3.3	10.2 +/- 0.8	53.8 +/- 2.8	13.4 +/- 0.7	73.2 +/- 3.8	12.6 +/- 0.9	88.4 +/- 3.2	6.1 +/- 1.0	23.0 +/- 1.7
rtL180M/sSilent + rtM204V/sI195M	Lamivudine	13.9 +/- 0.8	84.2 +/- 4.1	10.2 +/- 0.6	56.7 +/- 3.1	12.9 +/- 0.1	72.3 +/- 3.8	12.1 +/- 0.6	95.1 +/- 3.3	4.9 +/- 0.5	19.1 +/- 1.4
rtA181T/sW172STOP	Adefovir	0.9 +/- 0.2	-0.5 +/- 0.2	0.9 +/- 0.4	-2.1 +/- 0.2	0.6 +/- 0.1	-2.1 +/- 0.1	0.9 +/- 0.6	-0.2 +/- 0.1	0.9 +/- 0.1	-0.5 +/- 0.3
rtA181V/sL173F	Adefovir	11.8 +/- 0.8	79.3 +/- 3.6	10.9 +/- 0.2	56.1 +/- 3.4	13.4 +/- 0.7	83.7 +/- 4.1	12.0 +/- 0.6	88.3 +/- 3.0	7.2 +/- 1.0	27.4 +/- 1.4
rtT184S/sL176V	Entecavir	12.9 +/- 1.4	85.0 +/- 3.1	11.6 +/- 1.5	66.2 +/- 2.9	11.5 +/- 0.7	54.3 +/- 3.4	10.3 +/- 1.0	73.7 +/- 2.4	4.9 +/- 0.9	19.6 +/- 1.5
rtS202I/sV194F	Entecavir	12.9 +/- 0.7	90.7 +/- 3.4	10.5 +/- 0.7	62.0 +/- 2.8	11.1 +/- 0.4	56.0 +/- 3.6	10.3 +/- 0.7	74.3 +/- 2.2	5.9 +/- 0.7	27.6 +/- 1.5
rtS202I/sV194F + rtT184S/sL176V	Entecavir	1.0 +/- 0.2	-0.7 +/- 0.1	1.1 +/- 0.9	-1.0 +/- 0.1	0.5 +/- 0.2	-1.6 +/- 0.1	1.0 +/- 0.6	-2.5 +/- 2.1	1.1 +/- 0.4	0.6 +/- 0.1
rtS202I/sV194F + rtT184S/sL176V + rtI169T/sF161I	Entecavir	8.0 +/- 0.9	26.0 +/- 3.6	8.2 +/- 0.4	32.9 +/- 2.4	2.2 +/- 0.8	1.5 +/- 0.3	9.2 +/- 1.8	70.1 +/- 2.3	2.6 +/- 1.0	8.1 +/- 0.5
rtM204V/sI195M	Lamivudine	13.3 +/- 0.9	95.0 +/ 3.3	11.1 +/- 0.5	61.4 +/- 3.1	13.2 +/- 0.8	80.0 +/- 4.0	11.8 +/- 0.1	99.5 +/- 2.6	6.1 +/- 0.7	24.1 +/- 1.6
rtM204I/sW196S	Lamivudine	12.5 +/- 0.8	94.9 +/- 3.4	11.4 +/- 0.7	81.5 +/- 3.7	14.0 +/- 1.6	99.2 +/- 4.1	12.6 +/- 1.0	102.3+/- 3.1	6.2 +/- 1.2	26.3 +/- 1.6
rtM204I/sW196L	Lamivudine	12.7 +/- 0.4	83.1 +/- 3.7	10.0 +/- 0.5	53.6 +/- 2.9	13.2 +/- 0.9	71.3 +/- 4.0	11.8 +/- 1.1	83.0 +/- 3.3	6.4 +/- 1.0	19.2 +/- 1.8
Mean of positives	~	12.0 +/- 0.8	73.3 +/- 3.1	9.3 +/- 0.7	49.2 +/- 2.5	9.5 +/- 0.8	61.5 +/- 3.3	11.7 +/- 0.9	81.3 +/- 3.4	5.2 +/- 0.9	18.7 +/- 1.3

Table 4.1 – Summary of reactivity of wild type and all mutant HBsAg in all ELISAs used. AUC and N values which are less than the calculated +/- cut-off are highlighted in blue.

On the basis of the epitope balance data presented in table 4.1, each mutant HBsAg was sorted into one of the 8 theoretically possible phenotype groups based on MAb reactivity. These are displayed below in table 4.2.

Phenotype Group	P2D3	H3F5	D2H5
1	✓	✓	✓
2	✓	✓	X
3	✓	X	✓
4	✓	X	X
5	X	✓	X
6	X	✓	✓
7	X	X	✓
8	X	X	X

Table 4.2 – Possible phenotypic groupings of recombinant HBsAg according to epitope balance. There are potentially 8 possible phenotypes of which 5 were observed. Group 1 represents capture (marked with a red tick) with all MAbs, groups 2-4 represent loss of 2nd loop epitopes, and groups 5-8 represent lack of P2D3 reactivity.

The epitope balance data are presented in tables 4.3 and 4.4. As for table 4.2, data that are of questionable specificity due to low HBsAg expression (i.e., little or no P2D3 reactivity) have been tentatively placed in phenotype groups, but is considered in the context of low HBsAg levels. The results for this chapter are then discussed on the basis of these phenotypic groupings in sections 4.2 to 4.7. The horse polyclonal anti-HBs capture serum assay data are considered in section 4.8, and the results of the cell pellet lysate analysis in section 4.9. Finally, in the discussion section of this chapter, the

mutations are discussed as single, isolated mutations and then as the pairs of mutations studied. The wider implications of the data observed are considered in chapter 6.

		CAPTURE ANTIBODIES			
		P2D3 (121-129)	H3F5 (131-142)	D2H5 (142-147)	
Mutant	**Antiviral**	**Eptope Balance** (percentage of total MAb epitope binding +/- SD)			**Phenotype Group**
Wild Type	~	42.7 +/- 1.8	27.0 +/- 1.9	30.3 +/- 1.9	1
Wild Type - P2D3 excluded	~	~	47.2 +/- 3.2	52.8 +/- 3.2	
Wild Type - H3F5 excluded	~	58.5 +/- 2.4	~	41.5 +/- 2.6	
Wild Type - D2H5 excluded	~	61.2 +/- 2.5	38.8 +/- 2.8	~	
rtT128N/sP120T	Lamivudine	49.4 +/- 1.9	0.1 +/- 0.1	50.5 +/- 2.2	3
rtT128N/sP120T + rtM204V/sI195M	Lamivudine	59.8 +/- 2.7	0.0 +/- 0.1	40.2 +/- 2.6	3
rtSilent/sD144E	Lamivudine	44.2 +/- 1.6	27.7 +/- 1.5	28.0 +/- 1.3	1
rtSilent/sD144E + rtM204V/sI195M	Lamivudine	61.4 +/- 2.5	38.6 +/- 2.5	0.0 +/- 0.1	2
rtR153Q/sG145R	Lamivudine	82.2 +/- 4.1	17.8 +/- 1.1	0.0 +/- 0.1	2
rtR153Q/sG145R + rtM204V/sI195M	Lamivudine	65.6 +/- 3.7	35.4 +/- 2.3	0.0 +/- 0.1	2
rtR153K/sD144EsG145R	Lamivudine	76.0 +/- 8.3	24.0 +/- 1.9	0.0 +/- 0.1	4 ?
rtR153K/sD144EsG145R + rtM204V/sI195M	Lamivudine	97.3 +/- 5.3	2.7 +/- 0.3	0.0 +/- 0.1	4
rtF166L/sF158Y	Lamivudine	90.6 +/- 4.5	3.2 +/- 0.5	6.2 +/- 4.5	4
rtF166L/sF158Y + rtM204V/sI195M	Lamivudine	57.1 +/- 2.5	24.1 +/- 0.8	18.8 +/- 1.1	1
rtI169T/sF161L	Entecavir	55.8 +/- 2.1	40.2 +/- 2.4	4.0 +/- 0.4	2
rtV173L/sE164D	Lamivudine	46.1 +/- 2.2	24.0 +/- 1.3	30.0 +/- 1.9	1
rtV173L/sE164D + rtM204V/sI195M	Lamivudine	64.4 +/- 3.9	35.6 +/- 2.5	0.0 +/- 0.1	2
rtL180M/sSilent	Lamivudine	40.1 +/- 1.6	24.5 +/- 1.3	34.6 +/- 1.8	1
rtL180M/sSilent + rtM204V/sI195M	Lamivudine	39.5 +/- 1.9	26.6 +/- 1.5	34.0 +/- 1.8	1
rtA181T/sW172STOP	Adefovir	0.0 +/- 0.1	0.0 +/- 0.1	0.0 +/- 0.1	8 ?
rtA181V/sL173F	Adefovir	36.1 +/- 1.6	25.6 +/- 1.6	38.2 +/- 1.9	1
rtT184S/sL176V	Entecavir	41.4 +/- 1.5	32.2 +/- 1.4	26.4 +/- 1.8	1
rtS202I/sV194F	Entecavir	43.5 +/- 1.6	29.7 +/- 1.3	29.9 +/- 1.7	1
rtS202I/sV194F + rtT184S/sL176V	Entecavir	0.0 +/- 0.1	0.0 +/- 0.1	0.0 +/- 0.1	8 ?
rtS202I/sV194F + rtT184S/sL176V + rtI169T/sF161L	Entecavir	43.1 +/- 6.0	54.5 +/- 4.0	2.5 +/- 0.5	2
rtM204V/sI195M	Lamivudine	40.2 +/- 1.4	26.0 +/- 1.3	34.1 +/- 1.7	1
rtM204I/sW196S	Lamivudine	34.4 +/- 1.2	30.0 +/- 1.0	36.0 +/- 1.5	1
rtM204I/sW196L	Lamivudine	40.0 +/- 1.8	25.8 +/- 1.4	34.3 +/- 1.9	1

Table 4.3 – Relative performance capture MAbs with wild type and mutant HBsAg. AUC measured was combined for all three MAbs then each expressed as a percentage incorporating SD, allowing mutant MAb ELISA binding characteristics to be compared regardless of supernate HBsAg titre. Derived epitope density for pairs of MAbs were calculated by excluding one MAb from the balance analysis giving derivatised balance ratios. Mutants were grouped and colour coded according to phenotypic similarities in epitope balance (leaving those which are queried as colourless and annotated with a question mark) [see also table 4.4].

Phenotype Group	Antiviral	P2D3	H3F5	D2H5
1		√	√	√
Wild Type	~			
rtSilent/sD144E	Lamivudine			
rtF166L/sF158Y + rtM204V/sI195M	Lamivudine			
rtV173L/sE164D	Lamivudine			
rtL180M/sSilent	Lamivudine			
rtL180M/sSilent + rtM204V/sI195M	Lamivudine			
rtA181V/sL173F	Adefovir			
rtT184S/sL176V	Entecavir			
rtS202I/sV194F	Entecavir			
rtM204V/sI195M	Lamivudine			
rtM204I/sW196S	Lamivudine			
rtM204I/sW196L	Lamivudine			
2		√	√	X
rtSilent/sD144E + rtM204V/sI195M	Lamivudine			
rtR153Q/sG145R	Lamivudine			
rtR153Q/sG145R + rtM204V/sI195M	Lamivudine			
rtI169T/sF161L	Entecavir			
rtV173L/sE164D + rtM204V/sI195M	Lamivudine			
rtS202I/sV194F + rtT184S/sL176V + rtI169T/sF161L	Entecavir			
3		√	X	√
rtT128N/sP120T	Lamivudine			
rtT128N/sP120T + rtM204V/sI195M	Lamivudine			
4		√	X	X
rtR153K/sD144EsG145R ?	Lamivudine			
rtR153K/sD144EsG145R + rtM204V/sI195M	Lamivudine			
rtF166L/sF158Y	Lamivudine			
5		X	√	X
6		X	√	√
7		X	X	√
8		X	X	X
rtA181T/sW172STOP ?	Adefovir			
rtS202I/sV194F + rtT184S/sL176V ?	Entecavir			

Table 4.4 – Phenotypic groupings of wild type and mutant HBsAg according to epitope balance. There are potentially 8 possible phenotypes of which 5 are observed. Group 1 represents capture (marked with a red tick) with all MAbs, groups 2-4 represent loss of 2nd loop epitopes, groups 5-8 represent lack of P2D3 reactivity. Data that is queried is annotated with a question mark (?).

4.2: Epitope balance – Phenotype Group 1 – HBsAg capture with P2D3, H3F5 and D2H5

		CAPTURE ANTIBODIES			
		P2D3 (121-129)	H3F5 (131-142)	D2H5 (142-147)	
Mutant	**Antiviral**	**Eptope Balance** (percentage of total MAb epitope binding +/- SD)			**Phenotype Group**
Wild Type	~	42.7 +/- 1.8	27.0 +/- 1.9	30.3 +/- 1.9	1
Wild Type - P2D3 excluded	~	~	47.2 +/- 3.2	52.8 +/- 3.2	
Wild Type - H3F5 excluded	~	58.5 +/- 2.4	~	41.5 +/- 2.6	
Wild Type - D2H5 excluded	~	61.2 +/- 2.5	38.8 +/- 2.8	~	
rtSilent/sD144E	Lamivudine	44.2 +/- 1.6	27.7 +/- 1.5	28.0 +/- 1.3	1
rtF166L/sF158Y + rtM204V/sI195M	Lamivudine	57.1 +/- 2.5	24.1 +/- 0.8	18.8 +/- 1.1	1
rtV173L/sE164D	Lamivudine	46.1 +/- 2.2	24.0 +/- 1.3	30.0 +/- 1.9	1
rtL180M/sSilent	Lamivudine	40.1 +/- 1.6	24.5 +/- 1.3	34.6 +/- 1.8	1
rtL180M/sSilent + rtM204V/sI195M	Lamivudine	39.5 +/- 1.9	26.6 +/- 1.5	34.0 +/- 1.8	1
rtA181V/sL173F	Adefovir	36.1 +/- 1.6	25.6 +/- 1.6	38.2 +/- 1.9	1
rtT184S/sL176V	Entecavir	41.4 +/- 1.5	32.2 +/- 1.4	26.4 +/- 1.8	1
rtS202I/sV194F	Entecavir	43.5 +/- 1.6	29.7 +/- 1.3	29.9 +/- 1.7	1
rtM204V/sI195M	Lamivudine	40.2 +/- 1.4	26.0 +/- 1.3	34.1 +/- 1.7	1
rtM204I/sW196S	Lamivudine	34.4 +/- 1.2	30.0 +/- 1.0	36.0 +/- 1.5	1
rtM204I/sW196L	Lamivudine	40.0 +/- 1.8	25.8 +/- 1.4	34.3 +/- 1.9	1

Table 4.5 - Epitope Balance results for phenotype group 1.

Expressed as a percentage of all total MAb binding, the proportion of epitope binding for *in vitro*-generated wild type HBsAg was 42.7% +/- 1.8 against P2D3, 27.0% +/- 1.9 against H3F5 and 30.3% +/- 1.9 against D2H5. Eleven other mutant HBsAg supernates demonstrated a similar epitope balance to wild type HBsAg (table 4.5). This group included the 3 common lamivudine-resistance mutations in the YMDD motif - rtM204V/sI195M, rtM204I/sW196S and rtM204I/sW196L, which despite producing at times slightly higher N and AUC values (table 4.2), when expressed as a percentage showed no major shift in epitope balance. The common lamivudine compensatory mutation rtL180M/sSilent reacted like wild type HBsAg in the MAb capture ELISAs. Even upon the addition of the rtM204V/sI195M mutation to produce rtL180M/sSilent +

rtM204V/sI195M double mutation, the antigenic balance remained wild type-like. The raw AUC and N values were slightly less for all assays for the rtV173L/sE164D mutant (table 4.2), yet when the epitope balance was studied (table 4.4) it was clear that antigenic reactivity was wild type-like.

The adefovir resistance mutation rtA181V/sL173F, the entecavir resistance mutations rtT184S/sL176V and rtS202I/sV194F, as well as the putative vaccine-escape mutation rtSilent/sD144E all also resulted in wild type-like epitope balance ratios.

The most phenotypically distinct member of this group is the double mutant rtF166L/sF158Y + rtM204V/sI195M which showed a low epitope balance value for D2H5 of 18.8% +/- 1.1, compared to the value for wild type (30.3% +/- 1.9). It is of note that, as discussed in section 4.5, the single rtF166L/sF158Y mutation produced an epitope miss against both H3F5 and D2H5. However, the rtF166L/sF158Y + rtM204V/sI195M mutation also gave low results in the P2D3 ELISA, implying low HBsAg levels in the supernate. The unusual epitope balance results for the rtF166L/sF158Y + rtM204V/sI195M mutant could be artefactual as the capture assays were working with low titres of HBsAg close to the point of extinction, but despite this there was still capture by all MAbs.

Other than the rtF166L/sF158Y + rtM204V/sI195M mutation, all HBsAg mutations in this group were clearly detected in the ELISA using horse polyclonal anti-HBs capture ELISAs. Representative dilution curve data for this phenotype group 1 are displayed in figure 4.4 below, which shows dilution curves for all capture assays for the rtM204V/sI195M mutant.

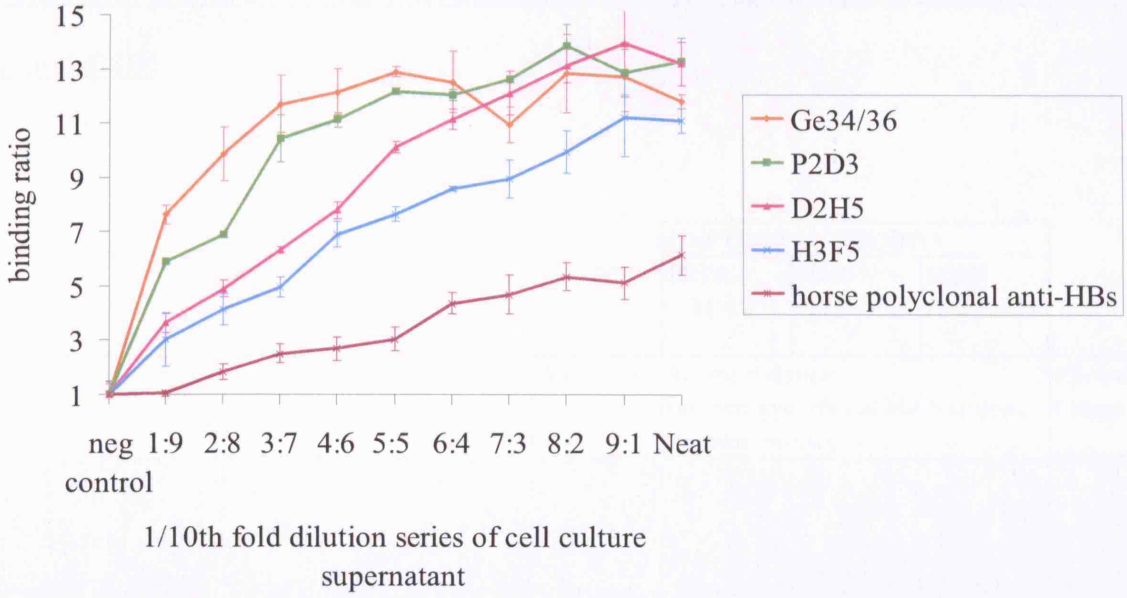


Figure 4.4: Mean binding ratios of mutant rtM204V/sI195M HBsAg in all MAb capture ELISAs that is representative of phenotype group 1.

4.3: Epitope Balance – Phenotype Group 2 - HBsAg capture with P2D3 and H3F5 but not D2H5

		CAPTURE ANTIBODIES			
		P2D3 (121-129)	H3F5 (131-142)	D2H5 (142-147)	
Mutant	**Antiviral**	**Eptope Balance** (percentage of total MAb epitope binding +/- SD)			**Phenotype Group**
Wild Type	~	42.7 +/- 1.8	27.0 +/- 1.9	30.3 +/- 1.9	1
Wild Type - D2H5 excluded	~	61.2 +/- 2.5	38.8 +/- 2.8	~	2
Wild Type - H3F5 excluded	~	58.5 +/- 2.4	~	41.5 +/- 2.6	3
Wild Type - P2D3 excluded	~	~	47.2 +/- 3.2	52.8 +/- 3.2	6
rtSilent/sD144E + rtM204V/sI195M	Lamivudine	61.4 +/- 2.5	38.6 +/- 2.5	0.0 +/- 0.1	2
rtR153Q/sG145R	Lamivudine	82.2 +/- 4.1	17.8 +/- 1.1	0.0 +/- 0.1	2
rtR153Q/sG145R + rtM204V/sI195M	Lamivudine	65.6 +/- 3.7	35.4 +/- 2.3	0.0 +/- 0.1	2
rtI169T/sF161L	Entecavir	55.8 +/- 2.1	40.2 +/- 2.4	4.0 +/- 0.4	2
rtV173L/sE164D + rtM204V/sI195M	Lamivudine	64.4 +/- 3.9	35.6 +/- 2.5	0.0 +/- 0.1	2
rtS202I/sV194F + rtT184S/sL176V + rtI169T/sF161L	Entecavir	43.1 +/- 6.0	54.5 +/- 4.0	2.5 +/- 0.5	2

Table 4.6 - Epitope Balance results for phenotype group 2.

This group of mutants (highlighted in orange in table 4.6) share the characteristics of loss of the D2H5 epitope, yet with no effect upon P2D3 and H3F5 epitopes.

As indicated in section 4.2, the single mutant rtV173L/sE164D and the single mutant rtM204V/sI195M share epitope balance properties with wild type HBsAg. However, the addition of rtM204V/sI195M to plasmid encoding rtV173L/sE164D results in the rtV173L/sE164D + rtM204V/sI195M double mutation which fails to bind to D2H5 (figure 4.7 below). Therefore, an interaction between the distal point mutations rtV173L/sE164D and rtM204V/sI195M appears to induce D2H5 epitope loss. The addition of the rtM204V/sI195M mutation to the sD144E/rtSilent mutation (each of which displays a wild type-like epitope balance) resulted in D2H5 epitope loss also.

This represents a second instance of the rtM204V/sI195M mutation interacting with distal point mutations to abrogate the D2H5 epitope.

The common vaccine escape mutation rtR153Q/sG145R demonstrates the most distinct phenotype of this grouping. It appears in addition to the loss of the D2H5 epitope to have a partial degree of epitope loss for MAb H3F5. Upon the addition of the rtM204V/sI195M mutation to the rtR153Q/sG145R backbone (generating the rtR153Q/sG145R + rtM204V/sI195M mutant) binding to H3F5 is restored and the double mutant resumes a more typical phenotype group 2 type of epitope balance. Thus this represents the third instance of distal mutational interaction, though here it leads to H3F5 restoration rather than D2H5 loss.

The single entecavir resistance mutation rtI169T/sF161L is also included in this phenotypic grouping. It produced a low binding ratio in the P2D3 assay and hence only expressed a low HBsAg titre in the supernate. The epitope balance presented could be an artefact of low HBsAg levels as all MAb capture assays were working very close to the point of extinction.

Representative data for phenotype group 2 are presented below in figure 4.5 and 4.6, which shows data for HBsAg rtV173L/sE164D. These data demonstrate the deletion of the D2H5 epitope upon combining both sE164D and sI195M mutations that in isolation react with D2H5.

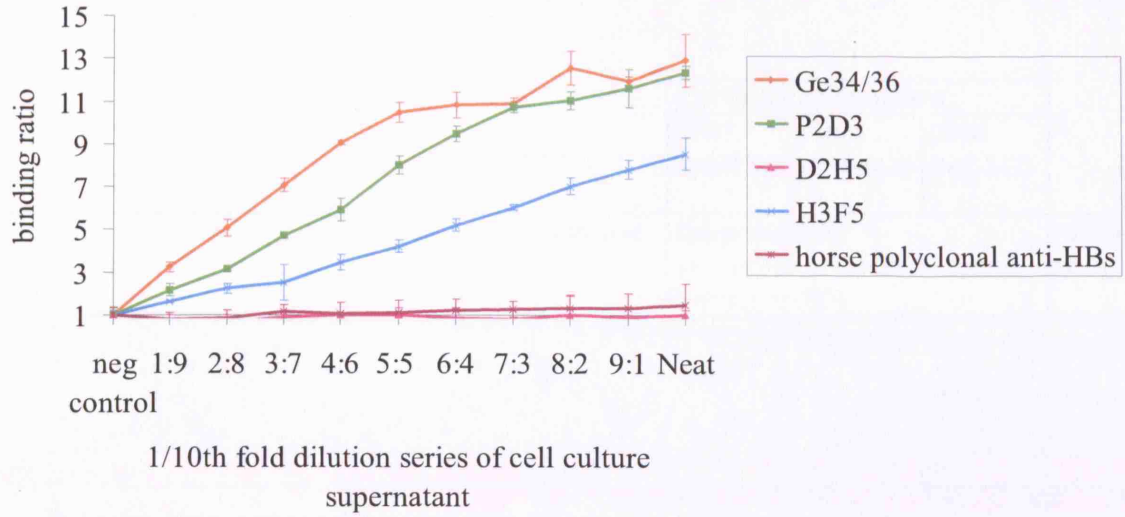


Figure 4.5 - Mean BRs of mutant rtV173L/sE164D HBsAg in all MAb capture ELISAs, which is representative of phenotype group 2.

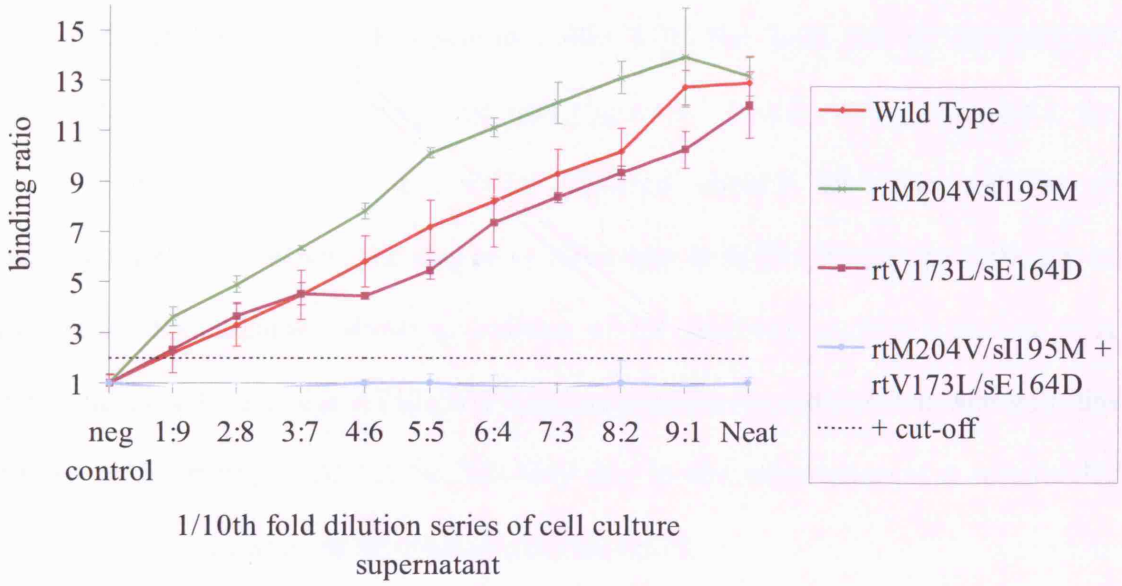


Figure 4.6 – Mean BRs of rtV173L/sE164D + rtM204V/sI195M mutant HBsAg in the D2H5 capture ELISA.

4.4: Epitope Balance – Phenotype Group 3 - HBsAg capture with P2D3 and D2H5 but not H3F5

		CAPTURE ANTIBODIES			
		P2D3 (121-129)	H3F5 (131-142)	D2H5 (142-147)	
Mutant	**Antiviral**	**Eptope Balance** (percentage of total MAb epitope binding +/- SD)			**Phenotype Group**
Wild Type	~	42.7 +/- 1.8	27.0 +/- 1.9	30.3 +/- 1.9	1
Wild Type - D2H5 excluded	~	61.2 +/- 2.5	38.8 +/- 2.8	~	2
Wild Type - H3F5 excluded	~	58.5 +/- 2.4	~	41.5 +/- 2.6	3
Wild Type - P2D3 excluded	~	~	47.2 +/- 3.2	52.8 +/- 3.2	6
rtT128N/sP120T	Lamivudine	49.4 +/- 1.9	0.1 +/- 0.1	50.5 +/- 2.2	3
rtT128N/sP120T + rtM204V/sI195M	Lamivudine	59.8 +/- 2.7	0.0 +/- 0.1	40.2 +/- 2.6	3

Table 4.7 - Epitope Balance results for phenotype group 3.

This phenotypic group of mutations consists of the rtT128N/sP120T mutation with or without the rtM204V/sI195M mutation (table 4.7). For both the rtT128N/sP120T mutant, there was clear H3F5 epitope loss (figure 4.7 below). When calculated, the epitope balances for P2D3 and D2H5 differed slightly after the addition of rtM204V/sI195M. However, the degree of difference is slight. Despite the difficulty in interpreting the outcome following addition of the rtM204V/sI195M mutation, it is interesting to note that the rtT128N/sP120T substitution is sufficient to abrogate the adjacent H3F5 epitope. This is presuMAbly due to the substitution of a structurally important proline residue (to be discussed in chapter 6).

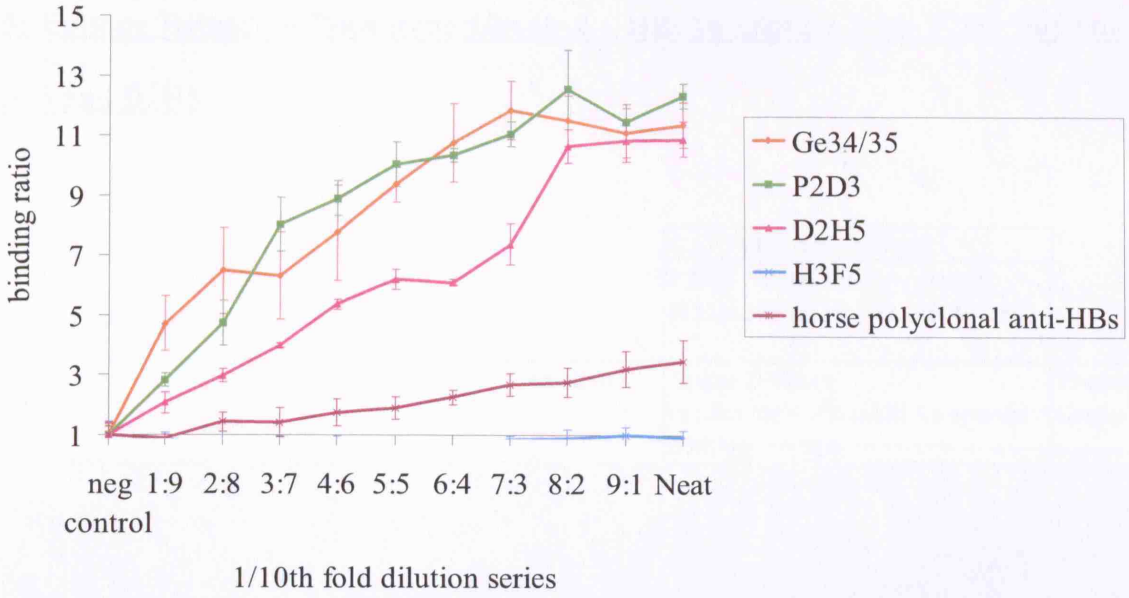


Figure 4.7 - Mean binding ratios of mutant rtM204V/sI195M + rtT128N/sP120T HBsAg in all MAb capture ELISAs, which is representative of phenotype group 3.

4.5: Epitope Balance – Phenotype Group 4 - HBsAg capture with P2D3 but not H3F5 and D2H5

		CAPTURE ANTIBODIES			
		P2D3 (121-129)	H3F5 (131-142)	D2H5 (142-147)	
Mutant	**Antiviral**	**Eptope Balance** (percentage of total MAb epitope binding +/- SD)			**Phenotype Group**
Wild Type	~	42.7 +/- 1.8	27.0 +/- 1.9	30.3 +/- 1.9	1
Wild Type - P2D3 excluded	~	~	47.2 +/- 3.2	52.8 +/- 3.2	
Wild Type - H3F5 excluded	~	58.5 +/- 2.4	~	41.5 +/- 2.6	
Wild Type - D2H5 excluded	~	61.2 +/- 2.5	38.8 +/- 2.8	~	
rtR153K/sD144EsG145R	Lamivudine	76.0 +/- 8.3	24.0 +/- 1.9	0.0 +/- 0.1	4 ?
rtR153K/sD144EsG145R + rtM204V/sI195M	Lamivudine	97.3 +/- 5.3	2.7 +/- 0.3	0.0 +/- 0.1	4
rtF166L/sF158Y	Lamivudine	90.6 +/- 4.5	3.2 +/- 0.5	6.2 +/- 4.5	4

Table 4.8 - Epitope Balance results for phenotype group 4.

This group of phenotypically similar HBsAg mutants is defined by clear P2D3 MAb capture, with H3F5 and D2H5 epitope loss. The rtR153K/sD144Es+G145R mutant is tentatively contained within this grouping on the basis of its epitope balance result. However the BRs obtained for it in the P2D3 capture ELISA were low. Again, any shifts in epitope balance observed may be artefactual due to the assays working with low HBsAg titres close to the point of extinction. Hence this mutant is not considered in any further analysis though there remains the possibility that it does demonstrate the profile of the H3F5 and D2H5 epitope loss.

Little comment can be made about the epitope balance of the two mutants (rtF158Y/sF161L and rtR135K/sD144E+sG145R) as they were only detected by P2D3 so no ratio can be presented. In both instances the P2D3 binding ratio was high and

hence the supernate HBsAg titre is high. It is striking that the rtF158Y/sF161L mutant is able to induce epitope loss of both the H3F5 and D2H5 epitopes. Representative data (rtF158Y/sF161L) for this phenotype grouping are presented below in figure 4.8.

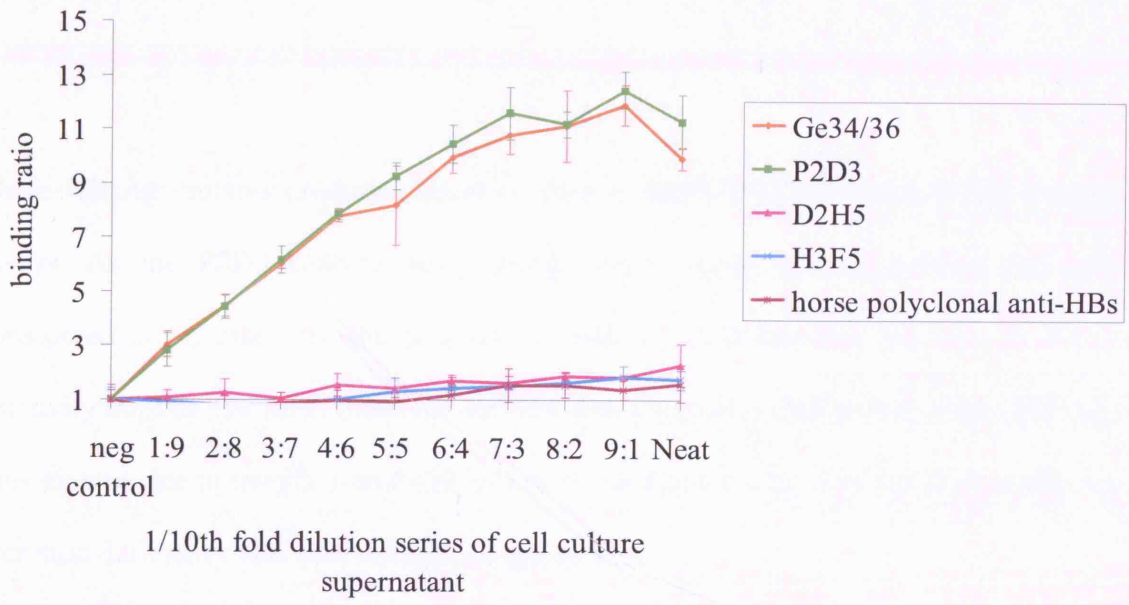


Figure 4.8 - Mean binding ratios of mutant rtF158Y/sF161L HBsAg in all MAb capture ELISAs, which is representative of phenotype group 4.

4.6: Epitope Balance – Phenotype Group 8 – no HBsAg capture with monoclonal antibodies P2D3, D2H5 and H3F5

		CAPTURE ANTIBODIES			
		P2D3 (121-129)	H3F5 (131-142)	D2H5 (142-147)	
Mutant	**Antiviral**	**Eptope Balance** (percentage of total MAb epitope binding +/- SD)			**Phenotype Group**
Wild Type	~	42.7 +/- 1.8	27.0 +/- 1.9	30.3 +/- 1.9	1
Wild Type - P2D3 excluded	~	~	47.2 +/- 3.2	52.8 +/- 3.2	
Wild Type - H3F5 excluded	~	58.5 +/- 2.4	~	41.5 +/- 2.6	
Wild Type - D2H5 excluded	~	61.2 +/- 2.5	38.8 +/- 2.8	~	
rtA181T/sW172STOP	Adefovir	0.0 +/- 0.1	0.0 +/- 0.1	0.0 +/- 0.1	8 ?
rtS202I/sV194F + rtT184S/sL176V	Entecavir	0.0 +/- 0.1	0.0 +/- 0.1	0.0 +/- 0.1	8 ?

Table 4.9 - Epitope Balance results for phenotype group 8.

Three HBsAg mutants produced negative BRs in the P2D3, H3F5 and D2H5 capture assays. As the P2D3 capture assay recognises a linear HBsAg epitope and was considered as an assay for the presence of HBsAg in supernates, the lack of P2D3 reactivity implies that these transfections failed to adequately express supernate HBsAg. This may be due to transfection failure, or as is considered in section 4.8, due to HBsAg secretion deficiency and intracellular accumulation.

These were the adefovir resistance mutation rtA181T/sW172STOP, the double entecavir resistance mutation rtS202I/sV194F + rtT184S/sL176V and the double vaccine escape mutation rtR153K/sD144EsG145R (table 4.2).

4.7: Horse polyclonal anti-HBs capture assay data ELISA outcomes

The inclusion of assays using the horse polyclonal anti-HBs assay was to control for the possibility of the reduction or loss of binding to the MAbs. However the data that emerged showed that there were many instances where mutant HBsAg did not also bind the horse polyclonal anti-HBs. This data must be considered carefully. In optimisation it was shown that the assay produces high background non-specific absorbances with negative control samples. When wild type HBsAg was assayed, a BR of just over 5 was generated. Typically to use an ELISA to reliably detect an antigen, a binding ratio of 10 is desirable. When polyclonal anti-HBs data was analysed, the DA HBsAg standard was always included as internal control at concentrations of 3 ng/ml HBsAg and 0.3 ng/ml. These data are presented in table 4.10 below. The analysis has been stratified into the phenotypic groupings.

Within phenotype group (wild type like) one, the mutant rtF166L/sF158Y is the only HBsAg mutant to produce poor results in the horse anti-HBs assay. The P2D3 AUC obtained for the same mutant is relatively low. Therefore, despite the apparent partial D2H5 epitope loss in the epitope balance analysis in conjunction with the poor horse anti-HBs capture assay result, it is difficult to make cogent comments about the epitope balance of this HBsAg

		CAPTURE ANTIBODIES					
		P2D3 (121-129)	H3F5 (131-142)	D2H5 (142-147)			
Mutant	**Antiviral**	**Eptope Balance** (percentage of total MAb epitope binding +/- SD)			**Phenotype Group**	**P2D3 AUC BR Result**	**Horse AUC BR Result**
Wild Type	~	42.7 +/- 1.8	27.0 +/- 1.9	30.3 +/- 1.9	1	85.1 +/- 3.5	19.8 +/- 1.4
rtSilent/sD144E	Lamivudine	44.2 +/- 1.6	27.7 +/- 1.5	28.0 +/- 1.3	1	85.7 +/- 3.0	16.2 +/- 1.4
rtF166L/sF158Y + rtM204V/sI195M	Lamivudine	57.1 +/- 2.5	24.1 +/- 0.8	18.8 +/- 1.1	1	40.8 +/- 1.8	2.3 +/- 0.3
rtV173L/sE164D	Lamivudine	46.1 +/- 2.2	24.0 +/- 1.3	30.0 +/- 1.9	1	77.0 +/- 3.6	14.0 +/- 1.3
rtL180M/sSilent	Lamivudine	40.1 +/- 1.6	24.5 +/- 1.3	34.6 +/- 1.8	1	85.0 +/- 3.3	23.0 +/- 1.7
rtL180M/sSilent + rtM204V/sI195M	Lamivudine	39.5 +/- 1.9	26.6 +/- 1.5	34.0 +/- 1.8	1	84.2 +/- 4.1	19.1 +/- 1.4
rtA181V/sL173F	Adefovir	36.1 +/- 1.6	25.6 +/- 1.6	38.2 +/- 1.9	1	79.3 +/- 3.6	27.4 +/- 1.4
rtT184S/sL176V	Entecavir	41.4 +/- 1.5	32.2 +/- 1.4	26.4 +/- 1.8	1	85.0 +/- 3.1	19.6 +/- 1.5
rtS202I/sV194F	Entecavir	43.5 +/- 1.6	29.7 +/- 1.3	29.9 +/- 1.7	1	90.7 +/- 3.4	27.6 +/- 1.5
rtM204V/sI195M	Lamivudine	40.2 +/- 1.4	26.0 +/- 1.3	34.1 +/- 1.7	1	95.0 +/ 3.3	24.1 +/- 1.6
rtM204I/sW196S	Lamivudine	34.4 +/- 1.2	30.0 +/- 1.0	36.0 +/- 1.5	1	94.9 +/- 3.4	26.3 +/- 1.6
rtM204I/sW196L	Lamivudine	40.0 +/- 1.8	25.8 +/- 1.4	34.3 +/- 1.9	1	83.1 +/- 3.7	19.2 +/- 1.8
rtSilent/sD144E + rtM204V/sI195M	Lamivudine	61.4 +/- 2.5	38.6 +/- 2.5	0.0 +/- 0.1	2	85.7 +/- 3.0	4.4 +/- 0.3
rtR153Q/sG145R	Lamivudine	82.2 +/- 4.1	17.8 +/- 1.1	0.0 +/- 0.1	2	68.5 +/- 3.4	0.1 +/- 0.1
rtR153Q/sG145R + rtM204V/sI195M	Lamivudine	65.6 +/- 3.7	35.4 +/- 2.3	0.0 +/- 0.1	2	67.5 +/- 3.8	16.4 +/- 1.3
rtI169T/sF161L	Entecavir	55.8 +/- 2.1	40.2 +/- 2.4	4.0 +/- 0.4	2	74.0 +/- 2.8	11.1 +/- 0.7
rtV173L/sE164D + rtM204V/sI195M	Lamivudine	64.4 +/- 3.9	35.6 +/- 2.5	0.0 +/- 0.1	2	63.0 +/- 3.8	1.4 +/- 0.2
rtS202I/sV194F + rtT184S/sL176V + rtI169T/sF161L	Entecavir	43.1 +/- 6.0	54.5 +/- 4.0	2.5 +/- 0.5	2	26.0 +/- 3.6	8.1 +/- 0.5
rtT128N/sP120T	Lamivudine	49.4 +/- 1.9	0.1 +/- 0.1	50.5 +/- 2.2	3	95.5 +/- 3.6	17.3 +/- 1.5
rtT128N/sP120T + rtM204V/sI195M	Lamivudine	59.8 +/- 2.7	0.0 +/- 0.1	40.2 +/- 2.6	3	76.5 +/- 3.5	10.5 +/- 0.8
rtR153K/sD144EsG145R + rtM204V/sI195M	Lamivudine	97.3 +/- 5.3	2.7 +/- 0.3	0.0 +/- 0.1	4	67.9 +/- 3.7	3.5 +/- 0.2
rtF166L/sF158Y	Lamivudine	90.6 +/- 4.5	3.2 +/- 0.5	6.2 +/- 4.5	4	72.1 +/- 3.6	0.9 +/- 0.3
rtR153K/sD144EsG145R	Lamivudine	76.0 +/- 8.3	24.0 +/- 1.9	0.0 +/- 0.1	4 ?	8.2 +/- 0.9	1.0 +/- 0.3
rtA181T/sW172STOP	Adefovir	0.0 +/- 0.1	0.0 +/- 0.1	0.0 +/- 0.1	8 ?	-0.5 +/- 0.2	-0.5 +/- 0.3
rtS202I/sV194F + rtT184S/sL176V	Entecavir	0.0 +/- 0.1	0.0 +/- 0.1	0.0 +/- 0.1	8 ?	-0.7 +/- 0.1	0.6 +/- 0.1

Table 4.10: horse polyclonal anti-HBs AUC data in comparison to MAb epitope balance and P2D3 AUC data.

For phenotype group 2 mutants (i.e., those associated with D2H5 epitope loss), almost all showed relatively high P2D3 AUC measurements. Such results may be considered more reliable than those with apparent low HBsAg titres. Thus, there are interesting contrasts to be made between the double mutations rtV173L/sE164D + rtM204V/sI195M and rtSilent/sD144E + rtM204V/sI195M as each led to poor capture by the hyperimmune serum, but all the single component mutations were captured by the same reagent.

The data obtained for rtR153Q/sG145R with or without rtM204V/sI195M are less clear. Again, both mutations produced good P2D3 AUC results and hence generated adequate levels of HBsAg in their supernate. However, whilst rtR153Q/sG145R was not detected by the polyclonal anti-HBs, the double mutant rtR153Q/sG145R + rtM204V/sI195M was detected. There was a shift in epitope balance to favour H3F5 upon the addition of rtM204V/sI195M to the rtR153Q/sG145R backbone. Thus, a single mutation could be associated with a potent effect in the hyperimmune serum capture assay.

The triple entecavir mutation rtS202I/sV194F + rtT184S/sL176V + rtI169T/sF161L within phenotype group 2 produced a low P2D3 AUC value and by inference, low HBsAg titres. Despite this, it was associated with a higher AUC value in the polyclonal anti-HBs assay.

Phenotype group 3 mutants (associated with H3F5 loss) produced sufficient P2D3 AUCs to be considered to have reliably expressed HBsAg. The rtT128N/sP120T + rtM204V/sI195M double mutant produced a poorer AUC in the horse polyclonal anti-HBs assay, though this may be explained in part by the likely lower levels of HBsAg in the supernate.

Phenotype group 4 mutants members rtR153K/sD144E+sG145R + rtM204V/sI195M and rtF158Y/sF166L (associated with H3F5 and D2H5 miss) both produced sufficient quantities of HBsAg (determined through P2D3 AUC) and yet produced very poor area under curve measurement for the horse polyclonal anti-HBs assay. Given that both these mutations readily abrogated both second loop epitopes, it seems likely that they abrogated the epitopes recognised by the horse anti-HBs reagent too. The mutation rtR153K/sD144E+sG145R produced too low a P2D3 AUC for there to be considered sufficient HBsAg in the supernate sample and so horse polyclonal ant-HBs reactivity cannot be commented on.

As per the mutation rtR153K/sD144E+sG145R, the two phenotype group 8 mutations, rtA181T/sW172STOP and rtS202I/sV194F+rtT184S/sL176V cannot be commented on due to low supernate HBsAg titres on the basis of the P2D3 AUC result.

4.8: Transfected CHO lysate binding results

Cell pellet lysates were subjected to non-denaturing lysis, using Tween 20 and IPEGAL non-ionic detergents that would not significantly disrupt the conformation of the membrane-bound HBsAg. Such experiments were a necessary control in order to demonstrate that there was no intracellular accumulation of HBsAg upon transfection due to secretion deficiencies or mutant HBsAg conformation. This was especially true in the instances where HBsAg in transfectant supernates was undetectable by P2D3.

The analysis of cell lysates makes it possible to determine if HBsAg was present internally in the cell. If HBsAg derived from lysates and supernates were to be both detectable, it could be concluded that normal secretion was occurring. Data from transfection with the wild type construct were used as benchmark and a supernate HBsAg/ lysate HBsAg concentration factor calculated. Such an approach had been used in a previous study (Jeantet *et al.,* 2004). However, if HBsAg were to be found contained internally within cells but not externally, then it could be concluded that the normal secretion of HBsAg had been inhibited.

The assay used to determine the presence of HBsAg was the Ge34/36 ELISA. As it contains P2D3 as a component monoclonal antibody, as per the supernate analysis, if HBsAg was present in the cell pellet lysate, then it ought to be detected by P2D3 (and hence Ge34/26) despite its conformation. There exists one caveat (as discussed in chapter 3): HBsAg titres produced upon transfection were low; therefore analysis of the wild type cell pellet lysate produced only a low BR of 2.37 in the Ge34/36 ELISA. Typically in ELISA, a signal-to-noise ratio of around 10:1 is considered acceptable for a positive standard. This ratio could not be obtained for wild type HBsAg lysates. The

level of intracellular HBsAg was at the limit of detection despite the pellets having been lysed in 1 ml lysis buffer as compared to 3 ml present for the supernates. Hence there was a three-fold increase in the stochiometry in favour of the pellet HBsAg. Accordingly, the assay was interpreted with caution considering the very low residual intracellular HBsAg titres observed.

Moreover, an important control is missing from this dataset. The non-ionic detergents Tween 20 and IPEGAL were present in the lysis buffer used on the cell pellets. The reactivity of known concentrations of the DA HBsAg in the presence of these reagents and in the Ge34/36 assay should have been more thoroughly investigated to determine if HBsAg remained detectable. However, ELISA diluent buffers often contain detergents, and the Tween 20 and IPEGAL may not have had a significant effect, but that remains undetermined and the omission of this control must be considered in interpreting the lysate binding ratio data.

The data are presented in table 4.11 below.

mutant	cell culture supernatant BR	cell pellet lysate BR	supernatant BR / lysate BR
wild type	11.64	2.37	4.91
mock transfection negative control	1	1	1
rtT128N/sP120T	11.33	2.85	3.98
rt128N/sP120T+rtM204V/sI195M	11.33	1.29	8.74
sD144E/rtSilent	11.47	1.95	5.88
rtSilentsD144E+rtM204V/sI195M	15.58	2.1	7.42
rtR153Q/sG145R	10.65	2.34	4.55
rtR153Q/sG145R+rtM204V/sI195M	11.94	1.86	6.42
rtR153K/sD144E + sG145R	3.34	1.01	3.35
rtR153K/sD144E+sG145R+rtM204V/sI195M	12.1	1.93	6.26
rtF166L/sF158Y	9.91	2	4.95
rtF166L/sF158Y+rtM204V/sI195M	9.52	1.87	5.09
rtI169T/sF161L	10	1.64	6.1
rtV173L/sE164D	11.43	1.65	6.93
rtV173L/sE164D+rtM204V/sI195M	12.89	1.89	6.81
rtL180M/sSilent	12.55	2.26	5.55
rtL180M/sSilent+rtM204V/sI195M	12.09	2.04	5.93
rtA181V/sS173F	11.95	3.03	3.94
rtA181T/sW172STOP *	0.92	2.12	0.43
rtT184S/sL176V	10.27	1.59	6.46
rtS202I/sV194F	10.3	1.73	5.95
rtS202I/sV194F + rtT184S/sL176V *	1.02	1.11	0.92
rtS202I/sV194F + rtT184S/sL176V + rtI169T/sF161L	9.29	1.91	4.86
rtM204V/sI195M	11.79	2.95	4
rtM204I/sW196S	12.61	3.16	3.99
rtM204I/sW196L	11.8	2.16	5.46

Table 4.11 – Wild type and mutant HBsAg supernatant and lysate binding ratios in theGe34/36 ELISA from cells transfected with mutagenised pBK-CMV[smallS]. Transfections which produced supernates which were negative for HBsAg are annotated (*).

All mutations other than rtR153K/sD144E+sG145R, rtA181T/sW172STOP and rtS202I/sV194F+rtT184S/sL176V produced supernatant/lysate factor results which were broadly consistent with wild type transfected cells, i.e., a ratio of 3.4:1 up to 9:1 of internal vs. external HBsAg concentrations. Hence, in all these instances, normal secretion with minimal intracellular accumulation is presumed to be occurring. As the assay was working at low end of its sensitivity, the variations in ratio should not be considered significant, except in those instances when the ratio becomes inverted and internal HBsAg concentration was higher than external HBsAg concentration.

4.8.1: rtR153K/sD144E+sG145R

The analysis of lysates from cells transfected with vector bearing the rtR153K/sD144E+sG145R double mutation produced a binding ratio of 1.01; thus, the background absorbance in this ELISA equalled the sample absorbance, and so no HBsAg was detectable in the pellet lysate sample. Whereas wild type transfectant cell lysates gave a binding ratio of 2.37, the transfectant supernate for the rtR153K/sD144E+sG145R mutation gave low BRs in the P2D3 capture ELISAs, but was nevertheless still detectable. Little can be inferred about the secretion efficiency of HBsAg produced in this transfection, as the data may reflect inefficient transfection.

4.8.2: rtA181T/sW172STOP

Lysate from CHO cells transfected with this mutant appeared to produce BRs that were higher than those detectable in the supernate, but was undetectable by all supernate assays (table 4.2). This outcome may be evidence of intracellular accumulation of HBsAg, but given the low binding ratios detected, further study would be necessary.

4.8.3: rtS202I/sV194F+rtT184S/sL176V

The lysate for cells transfected with this mutant plasmid showed no evidence of HBsAg as determined by the Ge34/36 ELISA (table 4.11). Mutant HBsAg in supernate from the same cell transfections was also undetectable (table 4.2). If the low supernate binding ratios observed were due to secretion inhibition, then HBsAg would be detectable in cell pellet lysates. This was not the case, but there was no detectable intracellular accumulation of HBsAg either. It is probable that the data reflect poor transfection.

4.9: Discussion.

From the above data antiviral resistance mutations may abrogate "a" determinant epitopes when each mutation acts alone and a pair of mutations act synergistically. Accordingly, the discussion below will be split along these lines. All single mutations will be considered in isolation; thereafter combinations of mutations will be dealt with.

4.9.1: Single mutations with no phenotypic effect

rtM204V/sI195M, rtM204I/sW196S and rtM204I/sW196L

These single mutants are associated with an epitope balance which is wild type-like. When assayed against MAbs known to bind to specific domains in the HBsAg "a" determinant, supernates from CHO cells transfected with pBK-CMV[smallS] constructs carrying the well-defined lamivudine resistance-associated mutations in the YMDD motif (rtM204V/sI195M, rtM204I/sW196S and rtM204I/sW196L) showed no shift in epitope balance characteristics. Nor was there any reduction in the BRs for YMDD mutants binding to polyclonal anti-HBs or in the commercially available Murex Ge34/36 HBsAg ELISA. These findings are not congruent with those of Torresi *et al.* (2002) who observed that such mutations were associated with reduced antigenicity in an ELISA using pooled vaccinee sera. They sought to explain their observations in terms of interaction of antibodies in the polyclonal anti-HBs pool with putative, downstream, externally-exposed epitope, which lie in the s187-207 region, one that is predicted by phage display studies to influence HBsAg binding (Chen *et al.,* 1996) [see figure 4.9 below].

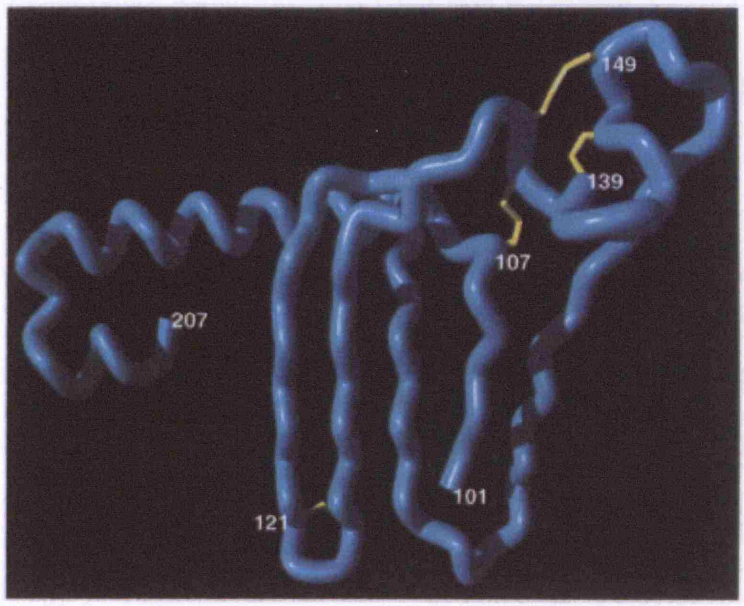


Figure 4.9 – A plausible structure for HBsAg. (Chen *et al.*, 1996).

My data do not substantiate this hypothesis, as the MAbs used in the current investigation do not bind to this epitope. However, no clear reductions in BRs in the horse hyperimmune serum assay for these mutants were observed, further suggesting that the three YMDD mutations studied do not detectably alter the conformation of the "a" determinant.

Moreover, Torresi *et al.* used hexahistidine tagging of their recombinant HBsAg in order to purify and then quantify it, allowing them to work with defined HBsAg concentrations. Their observations were based on the IC_{50} of HBsAg and pooled anti-HBs, i.e., the amount of HBsAg protein required to effect a 50% reduction in the binding to anti-HBs, and also in the reactivity of HBsAg in a radio-immunoassay.

In this thesis the alternative approach of studying epitope balance was adopted to reduce the effects of varying HBsAg titres. As epitope balance is a relative ratio, the HBsAg

titre is not significant, thus it can be considered that these mutations do not affect the expression of the "a" determinant. However, this is not true of the horse polyclonal anti-HBs data presented, which is the closest immunoassay presented in this thesis to that of Torresi and colleagues.

It is unknown if the data produced by Torresi and colleagues presented is based on single or multiple transfections and repeat assays. In contrast, the data presented in this thesis are based on a high degree of repetition of both transfection and assay, each AUC presented in table 4.1 being representative of 480 individual data points. Despite such repetition, the significance of changes in BRs relative to wild type is still interpreted with caution. By calculating the relative epitope balance for each mutant, the pitfall of varying HBsAg titres is avoided. Secondly, the P2D3 assay provided a semi-quantitive measure of HBsAg titre and so cases of very poor HBsAg expression could be identified. The epitope balance of the three YMDD mutations studied remained wild type-like, because there was clear capture in the P2D3 assay and the horse hyperimmune serum AUCs were greater than or equal to values for wild type HBsAg. Accordingly, the YMDD mutants (rtM204V/sI195M, rtM204I/sW196S and rtM204I/sW196L) do not significantly alter the antigenicity of HBsAg through abrogation of the "a" determinant. Lacking a MAb which binds the putative epitope at surface residues s187-207 and lacking sufficiently sensitive polyclonal anti-HBs capture assays we are unable to comment on the impact of the three mutations of the YMDD motif in this downstream epitope at surface residues s187-207.

rtL180M/sSilent

Study of the rtL180M/sSilent mutation was undertaken as a control to demonstrate that the process of site-directed mutagenesis did not alter antigenicity. In this instance, it was convenient that a control that was a lamivudine resistance-associated compensatory mutation could be chosen. The data produced for this mutant was, as expected, wild type-like in all assays and analysis, as the substitution was silent insofar as the S gene is concerned.

rtV173L/sE164D

Another lamivudine-associated compensatory mutation rtV173L/sE164D (Ogata *et al.,* 1999; Delaney *et al.,* 2003) was also studied and found to be wild type-like. It is of note that the raw AUC and N measurements were slightly less for all assays for rtV173L/sE164D mutant, yet when the epitope balance was studied it was clear that antigenic reactivity was wild type-like, thus demonstrating the value in the epitope balance approach, which can tolerate varying supernate HBsAg titres. If the AUC data was analysed for rtV173L/sE164D in the mode of Torresi *et al.* it would have been erroneously concluded that this mutation demonstrated a mild degree of epitope loss in all assays.

rtSilent/sD144E

The single rtSilent/sD144E mutation was studied, as it represents a vaccine-escape mutation, often observed in conjunction with sG145R (Torresi *et al.,* 2002b). It had been considered to be a possible compensatory mutation in conjunction with rtR153K/sG145R but that eventually was shown was not to be the case (Torresi *et al.,* 2002a). This mutant, however, was not observed to abrogate any second loop epitopes.

rtA181V/sW173F, rtS202I/sV194F and rtT184S/sL176V

There is no evidence for any altered HBsAg antigenicity associated with the adefovir resistance mutation rtA181V/sW173F and it is also clear that the two entecavir resistance-associated mutations - rtS202I/sV194F and rtT184S/sL176V - do not lead to any alteration in HBsAg epitope balance. The study of these mutations in isolation may be academic; however, as entecavir resistance seems to be principally effective when these mutations are present in conjunction with the lamivudine resistance-associated mutations rtM204V/sI195M, rtV173L/sE164D and rtL180M/sSilent (Tenney et al, 2004; Villet *et al.*, 2007).

4.9.2: Single mutations with clear phenotypic effect

rtT128N/sP120T

This mutant is a striking example of a single mutation that alters phenotype. More recently this lamivudine resistance-associated compensatory mutation has been appreciated as contributing to adefovir resistance as well (Stephen Locarnini – personal communication, 2007). The current studies show complete epitope loss for H3F5 whose epitope lies in residues s131-142. It is of note that the mutation leads to a replacement of proline. Proline residues are significant contributors to protein conformation and often found at the end of an α helix or in turns or loops. The radical epitope loss may thus be explained. Nonetheless, it is interesting that binding to the epitope adjacent to the site of substitution s121-129) remains unaffected, whilst the distal second loop is altered. As this mutation is considered a compensatory change following lamivudine resistance it is unlikely to arise in isolation as a result of antiviral therapy but in conjunction with a primary lamivudine resistance mutation. Thus, what the epitope

balance is for rtT128N/sP120T following interaction with rtM204V/sI195M would be more clinically relevant.

rtR153Q/sG145R

The mutant sG145R has long been recognised as a vaccine-escape mutation (Carman *et al.,* 1990), but more recently it has been appreciated also as a lamivudine resistance-associated compensatory mutation due to an rtR153Q substitution in *pol* (Torresi *et al.,* 2002a). The supernate carrying the rtR153Q/sG145R mutant was reduced in its reactivity to H3F5, albeit not completely, as AUC and N values were both above cut-off. There was complete epitope miss for D2H5. It is not surprising that a common immune escape mutation should lead to abrogation of second loop "a" determinant epitopes and it has been documented that the P2D3 epitope remains unperturbed by it (Ijaz *et al.*, 2003). The data serves as a useful comparison, as immune escape by sG145R is a well understood phenomenon, and by contrasting data on immune escape by selection of nucleotide resistance mutations in the overlapping gene to sG145R, a better appreciation of the degree of the phenomenon is afforded.

rtF166L/sF158Y

The rtF166L/sF158Y mutation is located in the putative fingers sub-domain of *pol* and is considered a lamivudine resistance-associated compensatory mutation. In isolation, it produced a striking epitope balance that abrogates both the D2H5 and H3F5 epitopes despite the substitution being located 11 and 16 amino acids distant from the epitope. However, as with rtT128N/sP120T, this mutation has only been described as a lamivudine-associated compensatory mutation and it is unknown if it would emerge in isolation *in vivo*. Although the data for the single mutation is interesting, the interaction of it with primary lamivudine resistance mutations remains the more clinically relevant.

rtA181T/sW172STOP

In the case of adefovir resistance, there is firm evidence of secretion inhibition for the rtA181T/sW172STOP mutation. Truncated HBsAg is known to be secretion deficient (Bruss and Ganem, 1991, Jenna & Surreau 1999), as although the localisation signals at the amino terminus of the HBsAg protein direct the truncated protein to the ER, secretion may not occur owing to the protein having adopted the wrong conformation due to misfolding; this misfolding can result in retention in the ER membrane. A large proportion of truncated S protein is also not glycosylated (Jenna & Surreau 1999), which may again affect the folding and hence the secretion characteristics of truncated protein. It is possible that the accumulation of such a mutation in the HBV genome may be physiologically relevant. Some studies have sought to implicate HBV genomes bearing stop codons in this region of the small S gene as the cause behind a more aggressive and fulminant disease course in infected patients (Gerner *et al.,* 2003; Yang & Tang *et al.,* 2003). It is unclear if virus bearing such a genome is fully viable. The possibility exists that HBsAg derived from other members of the quasispecies population infecting the individual not bearing this mutation had been used with the wild type virus in the quasispecies population acting as "helper virus", a phenomena known as complementation (Okamato *et al.*, 1993).

rtI169T/sF161L

The rtI169T/sF161L mutation was originally described as a putative entecavir resistance mutation having been found in patients demonstrating phenotypic resistance to entecavir. It appears that this mutation induces maximum antiviral resistance in conjunction with the mutations rtV173L/sE164D and rtM250V (Tenney *et al.*, 2004;

Villet *et al.*, 2007). Hence the phenotype observed for rtI169T/sF161L may not be found to arise in isolation *in vivo*. The mutation causes D2H5 epitope miss, despite being located at least 14 amino acids distant from the epitope, demonstrating the potential of such mutations to affect distal sites. It is unknown how this mutation would interact with the rtV173L/sE164D mutation with which it is associated as these experiments were not conducted in this study.

4.9.3: Combination mutations

There exist 4 categories of combination mutations; (i) those whose data cannot be commented on due to very low HBsAg titres, (ii) those which demonstrate no additive phenotypic effect, (iii) those which possibly demonstrate mild additive phenotypic effect and (iv) those which demonstrate a clear additive effect. They will be dealt with in that order.

Transfections which produced low HBV Titres

rtR153K/sD144E+sG145R

As discussed, the HBsAg titre for the rtR153K/sD144E+sG145R mutation could be the result of a poor transfection and there was no evidence of intracellular accumulation of HBsAg. The epitope balance data presented may be an artefact of poor HBsAg expression and so the data cannot be meaningfully commented on.

rtS202I/sV194F+rtT184S/sL176V

The double entecavir mutation rtS202I/sV194F + rtT184S/sL176V also did not to produce any detectable HBsAg as evidenced in its negative P2D3 assay result, nor was there any evidence of intracellular accumulation of HBsAg. It is inferred that this outcome was a result of a bad transfection. The plasmid was engineered by adding the rtT184S/sL176V mutation onto plasmid already containing the rtS202I/sV194F mutation, and the rtS202I/sV194F plasmid later proved to be functional for the production of HBsAg.

rtS202I/sV194F + rtT184S/sL176V+rtI169T/sF161L

This triple entecavir mutant did produce detectable HBsAg as demonstrated in the P2D3 capture assay. However, the BR was much lower than for all other positive supernates. This low, but not completely negative HBsAg titre meant that the significance of data generated from this double mutant needs to be circumspect. Although there are parallels between this data set and that for the single mutation rtI169T/sF161L in regard to the loss of the D2H5 epitope, caution must be exerted not to over interpret what may be an artefact of low HBsAg titre.

Combination mutations with no additive phenotypic effect

rtT128N/sP120T+rtM204V/sI195M

The rtT128N/sP120T mutation is an example of a resistance compensatory mutation that can abrogate the H2F5 epitope *per se*. It was important to study the double rtT128N/sP120T+rtM204V/sI195M mutant as a resistance compensatory mutation would always be coupled with a primary resistance mutation in a clinical setting. At first glance there appears to be little difference upon the addition of rtM204V/sI195M as the epitope balance still shows clear H3F5 epitope loss. The AUC data for D2H5 does appear to show that there is a slight alteration in epitope balance resulting in a mild degree of epitope loss for D2H5. However, this effect is likely artefactual. Although the neat binding ratios N for rtT128N/sP120T with and without rtM204V/sI195M are similar, the AUC value is dramatically smaller for rtT128N/sP120T + rtM204V/sI195M and so the possibility that the reduced D2H5 AUC is an artefact cannot be discounted.

rtL180M/sSilent + rtM204V/sI195M

Neither the rtL180M/sSilent nor the rtM204V/sI195M mutants in isolation affect the epitope balance. As discussed above, the rtL180M/sSilent mutant was included as a control and the same is true for rtL180M/sSilent+rtM204V/sI195M mutant. As there is no change on the amino acid level to differentiate it from rtM204V/sI195M the pattern of epitope balance observed is like that of rtM204V/sI195M and hence wild type-like.

Combination mutations with possible phenotypic effect

rtR153Q/sG145R+rtM204V/sI195M

The sG145R mutation may be considered a lamivudine resistance-associated compensatory mutation due to an rtR153Q substitution in *pol* (Torresi *et al.,* 2002a). Therefore it had to be studied in combination with a primary lamivudine resistance-associated mutation, rtM204V/sI195M. In isolation, the rtM204V/sI195M mutation produced a wild type-like epitope balance, and the rtR153Q/sG145R mutation resulted in complete loss of the D2H5 epitope and partial loss of the H3F5 epitope. The addition rtM204V/sI195M to rtR153Q/sG145R generated no influence on the D2H5 epitope, yet there is evidence of a mild partial restoration of the H3F5 epitope, the AUC having more than doubled. This phenomenon is striking. It implies that if HBsAg bearing the prototypic immune escape mutation sG145R then acquires the prototypic lamivudine resistance-associated mutation, that it may become more readily detectable by anti-HBs. Whether this *in vitro* finding is likely to be relevant *in vivo* is discussed in conjunction with other mutations in Chapter 6.

rtF166L/sF158Y+rtM204V/sI195M

The mutation rtF166L/sF158Y + rtM204V/sI195M produced supernates which gave significantly lower P2D3 AUC measurements, hence less HBsAg was present in these samples and the data must be treated cautiously. A reduced HBsAg titre has been enough to discount other mutants from this analysis. Yet the addition of rtM204V/sI195M to the lamivudine compensatory mutation rtF166L/sF158Y resulted in increased AUC for the H3F5 assay and a small increase in AUC in the D2H5 assay. It is unlikely that these would be the artefactual effects of a reduced HBsAg titre. When this data set is interpreted in the context of epitope balance, a partial restoration of the

H3F5 and D2H5 epitopes is apparent, but not conclusive on account of the low HBsAg levels in the supernate.

Combination mutations with clear additive phenotypic effect

rtR153K/sD144E+sG145R+rtM204V/sI195M

This triple mutant did produce sufficient HBsAg titres unlike the rtR153K/sD144E+sG145R double mutant. Its epitope balance reflected P2D3 capture only, thus both second loop epitopes had been abrogated. If this outcome is contrasted to that for the rtR153Q/sG145R+rtM204V/sI195M mutant, the addition of the sD144E mutation could be seen to exert an influence on the rtR153Q/sG145R+rtM204V/sI195M backbone as there is clear capture by H3F5. The addition of the sD144E mutation in the H3F5 epitope to rtR153Q/sG145R+rtM204V/sI195M goes on to affect the adjacent D2H5 epitope (s142-147).

rtV173L/sE164D+rtM204V/sI195M

The common lamivudine resistance-associated compensatory mutation, rtV173l/sE164D (Ogata *et al.,* 1999; Delaney *et al.,* 2003) plus the rtM204V/sI195M mutation when combined yielded a very interesting pattern of results; the mutations interact to abrogate epitopes on the second loop of the "a" determinant. Whilst the rtV173l/sE164D mutation demonstrated a wild type-like epitope balance, the addition of rtM204V/sI195M mutation resulted in D2H5 epitope loss and reduced HBsAg binding to horse hypermmune serum. This set of results corroborates the result that Torresi *et al.* (2002) obtained. They observed that the rtV173L/sE164D+rtM204V/sI195M double mutant was as poorly recognised by pooled vaccinee sera as the vaccine-escape sG145R

mutant. My data characterise this double mutant to abrogate the D2H5 epitope, i.e., at s142-147. It is proposed that the abrogation of this epitope in the second loop of the "a" determinant is the cause of the observations made by Torresi and colleagues.

It has been observed before that amino acid substitutions in the HBsAg protein outside the "a" determinant can cause epitope change within the "a "determinant (Carman *et al.,* 1997; Terrault *et al.,*, 1998; Oon *et al.,*, 1999; Kfoury-Baz *et al.,* 2001; Jeantet *et al.*, 2004; Wagner *et al.*, 2004) and that changes in the 1st loop of the "a" determinant can trigger epitope change within the 2nd loop (Waters *et al.,*, 1992). However, "epitope moderation" has not been described over such a large distance in the HBsAg protein, nor through the co-operative interaction of 2 distinct amino acid substitutions. For the rtV173L/sE164D+rtM204V/sI195M mutant, the point mutations are situated 31 amino apart, yet alter the second loop of the "a" determinant which is 17-48 amino acids upstream. This observation suggests that protein folding might have occurred in such a way as to bring the 2 regions more closely together than predicted by current models of the HBsAg protein. Torresi et al (2002) sought to explain their findings through an abrogation of epitopes at surface residues s187s207. The data presented here would suggest that abrogation of the the "a" determinant itself is sufficient. In any case, for pooled vaccinee sera, the majority of antibodies present would be directed against the "a" determinant (Howard *et al.*, 1984).

rtSilent/sD144E+rtM204V/sI195M

Epitope moderation was also observed for the rtSilent/sD144E+rtM204V/sI195M double mutant. Although the sD144E mutant used in this study was silent in the *pol* gene, compensatory nucleotide substitutions in the putative fingers subdomain of RT (Das *et al.,* 2001) have been described in *pol* that lead to the sD144E mutation (Torresi

et al., 2002a). While the rtSilent/sD144E mutant was captured by all the antibody reagents used, the addition of rtM204V/sI195M mutation to produce rtSilent/sD144E + rtM204V/sI195M double mutation resulted in a complete loss of D2H5 binding. The same pattern of reduced antigenicity was also observed for the rtV173L/sE164D+rtM204V/sI195M double mutation (discussed above). In this instance the interaction of the 2 mutations was over 51 amino acids apart and affected an epitope that was distant from the individual point mutations, one mutation being 48 amino acids away. This second instance of distal epitope moderation by two amino acids substitutions again is suggestive of protein folding of HBsAg not explained by current topological models.

In summary, this chapter shows confirmatory and novel data revealing that mutations associated with resistance to currently licensed drugs for the treatment of chronic hepatitis B can alter the antigenicity of HBsAg through changes in and outside the "a" determinant. Some of the data presented suggests our understanding of the conformation of downstream regions of HBsAg may be incomplete. Further, normal secretion of HBsAg may also be affected by antiviral resistance mutations. As the drugs become more widely used, diagnostic and clinical concerns arise. The wider implications of the data presented in this chapter are discussed further in Chapter 6.

CHAPTER 5

MOLECULAR EPIDEMIOLOGY OF ACUTE HBV INFECTION IN ENGLAND, 1997-2001

5.1: Introduction

Since 1977, HBIG has been administered to babies born in the UK to HBeAg-seropositive mothers as a means of providing passive immunity. From 1979, passive hepatitis B prophylaxis was complemented by vaccination of babies with hepatitis B vaccine to provide active immunisation. Vaccine-escape variants may emerge through such practices, particularly when prophylaxis is given incompletely or delayed (Carman *et al.,* 1990). The mutants may also arise in individuals receiving HBIG therapy (Protzer-Knolle *et al.,* 1998; Ghany *et al.,* 1998; Carman *et al.,* 1996), which is used before liver transplantation to suppress the viral load.

Since 1998, lamivudine for the treatment of chronic hepatitis B gained licensure. With prolonged therapy, mutants in the *pol* gene were found to emerge. These have potential to cause mutations in the overlapping S gene (Torresi *et al,.* 2002; Bock *et al.,* 2002). Such mutants have been reported to be horizontally transmissible (Chakravarty *et al.* 2002; Ho *et al.,* 1995; Ogata *et al.,* 1997; Thibault *et al.,* 2002), and capable of producing disease (Ogata *et al.,* 1997, Thibault *et al.,* 2002).

Phylogenetic analysis has been used to study evolutionary relationships. In early studies, phylogenetic trees were constructed based on gene frequency data and morphological characteristics (Weiller *et al.,* 1995). Phylogenetic trees are visual representations of empirically calculated evolutionary relationships between individual organisms. Since the introduction of PCR amplification and subsequent widespread use of DNA sequencing, it has been possible to perform phylogenetic analysis of DNA sequences from a large number of sources, including pathogens such as HBV. Phylogenetic analysis of nucleotide sequence data has been employed to track the spread of such pathogens in populations, to infer their evolutionary relationships, and to

facilitate HBV typing (Okamoto *et al.*, 1988; Sugauchi *et al.*, 2003a; Norder *et al.*, 2004).

For HBV, studying the evolutionary relationships of subgenomic DNA sequences can prove useful. Comparisons of sample sequences to defined sequences of known genotype may allow a novel sequence to be genotyped based on the degree of homology to prototypes. Predicting evolutionary relationships between DNA sequences is also useful when done in conjunction with epidemiology. If the viral genomic or sub-genomic sequence isolated originates from a particular geographic location or risk factor group (such as MSM), the coupling of epidemiological to molecular phylogenetic data allows the spread of the virus to be tracked (Ngui *et al.,* 1997). Phylogenetic analysis of viral sequences is also useful in defining transmission events. By comparing sequences isolated from the recipient to that of the suspected transmitter against a background of local sequences, transmission from the former to the latter may be inferred (Webster *et al.,* 2000).

The Health Protection Agency (HPA) as a tertiary reference centre receives a large number of patient serum samples each year for testing of markers of infection with HBV, many of which are from patients with acute hepatitis B. It was previously observed that study of laboratory notes and samples sent for laboratory analysis gives an accurate estimate of the incidence of symptomatic disease within the population and is a useful approach to evaluating disease trends (Polakoff & Tillet, 1984).

The aim of the study described in this chapter was to use phylogenetic analysis in conjunction with an assessment of risk factors to study the role of iatrogenically induced HBV mutants in acute hepatitis B. Serum samples from patients referred to the HPA in

the period between1997 and 2001, which corresponds to a period in which immunisation programmes with HBIG and hepatitis B vaccine were well established and antiviral therapy with lamivudine was just being introduced.

5.2: Sample selection

Acute hepatitis B was identified serologically on the basis of positive serology for HBsAg and an anti-HBc IgM level of >200 PEIU/ml. The HPA Sexually transmitted and Blood-Borne Virus Laboratory serum archive was sourced for patients' samples meeting this definition. Of 427 samples identified, 253 were found to contain sufficient serum volume for molecular studies. Samples tended to be referred from hospitals or clinics in south-east England (Hallet *et al.,* 2004).

The mean age of patients at time of sampling was 36.0 years, the youngest patient was < 1 month old, and the oldest noted patient was 87, thus giving a median age of 43.5 years.

The previously determined HBeAg and anti-HBe serostatus was also recorded for all patients, this is recorded in table 5.1 below.

HBeAg status	anti-HBe status	% of total patients
HBeAg +	anti-e -	64%
HBeAg -	anti-e +	17.7%
HBeAg +	anti-e +	1.7%
HBeAg -	anti-e -	0.8%
HBeAg eqivocal	anti-e -	5.9%
HBeAg -	anti-e equivocal	4.2%
HBeAg equivocal	anti-e equivocal	0.7%
HBeAg not determined	HBeAg not determined	7.6%

Table 5.1 – HBeAg and anti HBe status for 160 PCR-positive serum samples

5.3: Extraction of DNA

The NIBSC HBV DNA quantitative serum standard (Saldanha *et al.,* 2001) was used to allow the automated robotic MagNA Pure (Roche) extraction method to be compared with the method based on the QIAamp DNA Blood Mini Kit (Qiagen) manual spin column. The NIBSC HBV DNA standard was provided at 10^6 International Units/ml (IU/ml). This was diluted to concentrations of 10^5, 10^4, 10^3, 10^2 and 10^1 IU/ml. 100 µl from each concentration was then extracted under standard conditions as instructed by the manufacturer. The extracted DNA underwent PCR in optimised conditions with the use of EXPAND (Roche) DNA polymerase. Agarose gel electrophoresis allowed assessment of which concentrations had been successfully amplified from each DNA extract (figure 6.1). The results showed that QIAamp was as sensitive as MagNA Pure, both leading to PCR sensitivity of 10^2 IU/ml. Since all the samples to be assayed were from patients likely to be highly viremic (Dane *et al.,* 1970; Busch & Kleinman 2000), a PCR protocol attaining a sensitivity of 10^2 IU/ml applied to 200 µl of serum was considered sufficient to amplify 10^9 genome-equivalents/ml (Saldahna *et al.,* 2001) to permit downstream sequencing analyses. The large number of samples that required to be extracted necessitated the adoption of MagNA Pure over QIAamp.

5.4 PCR: amplification

The PCR amplification methods outlined in Section 2.7.5 were applied. Examples of the data obtained are presented in figure 5.1 and figure 5.2 below.

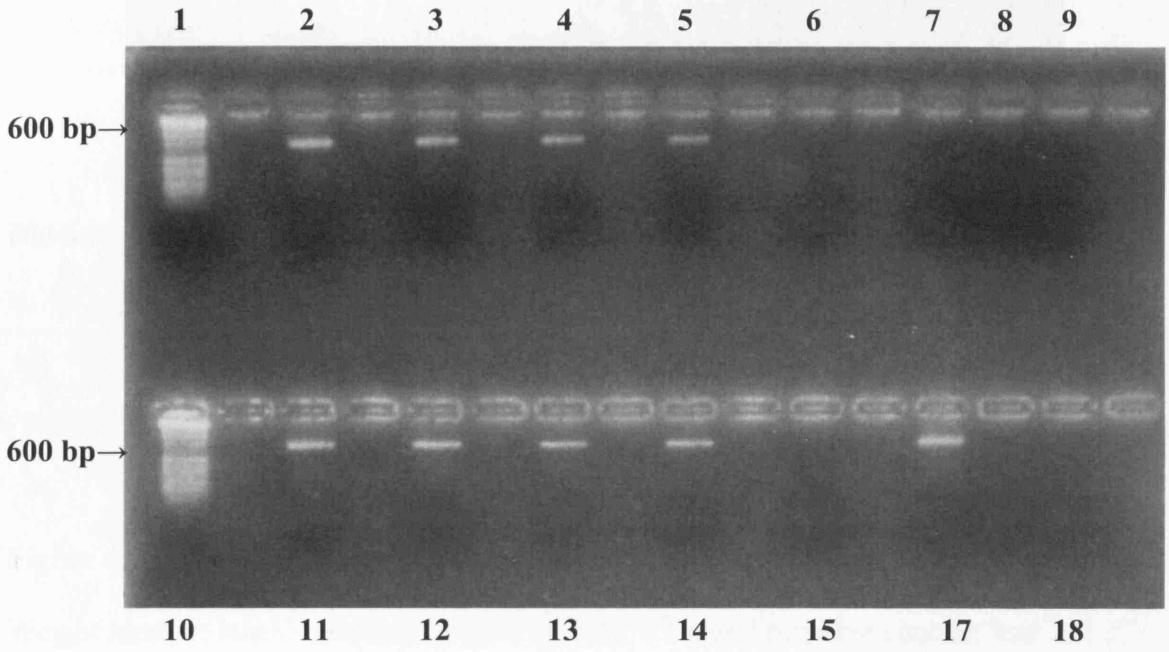


Figure 5.1 – Comparison of PCR sensitivity between MagNA Pure (Roche) and QIAamp Blood Mini Kit (Qiagen) extraction.

Lanes 1 and 10: molecular weight marker; lanes 2-6: NBSC standard at 10^5-10^1 IU/ml extracted using MagNA Pure (Roche); lanes 11-15: NBSC standard at 10^5-10^1 IU/ml extracted using QIAamp (Qiagen); lanes 7 and 8: extraction negative control; lane 17: PCR positive control; lane 18: 2nd round PCR negative control.

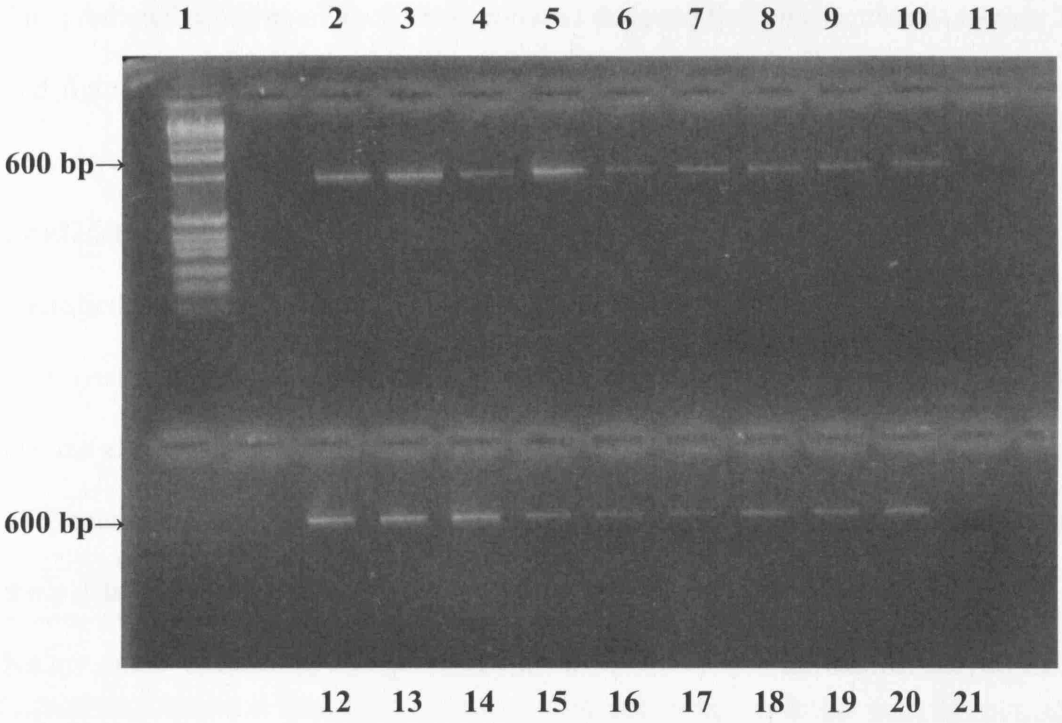


Figure 5.2 – Sample results from nested PCR for HBV S gene. Lane 1: Molecular Weight Marker; lane 2: positive control; lane 11: 1st round negative control; lane 21: 2nd round negative control; lanes 3-20: test wells containing second round PCR product.

5.5: Sequencing results

PCR amplicons from 160 samples could be generated from which HBV sub-genomic sequences were obtained (see materials and methods 2.7.7). Each amplicon encompasses the first 600 bases of the 682-bp small S gene. The sequences were aligned against a panel of GenBank sequences derived from known genotypes and were checked for the presence of iatrogenic mutations as previously described in the literature (Cooreman *et al.,* 2001; Locarnini *et al.,* 2003; Fung & Lok, 2004; Zoulim, 2004). No mutations in either *pol* or the S gene were detected. No mixed genotype infections could be identified from the chromatographic data.

5.6: Predicted Serotype analysis

The predicted serotype of each amplicon was deduced from its sequence (section 2.2.13 and figure 2.5). From the sequenced amplicons, 95/160 (59.4%) could be predicted to bear serotypes: *adw2*, *adrq+* 8/160 (5%); *ayw1* 5/160 (3.1%); *ayw2* 9/160 (5.6%); *ayw3*26/160 (16.2%); and *ayw4* 4/160 (2.5%). Two variants (2/160 [1.3%]) were identified to possess a glutamine at codon 122: hence the *d/y* type could not be deduced. One *ayw2* variant with an unresolved *q* determinant was also identified: it possessed an alanine at codon 177 of the S gene, specifying *q-*, but also possessed a proline at codon 178, specifying *q+*; therefore it was impossible to predict whether this variant carried the *q* determinant or not. All other samples were predicted to belong to the *q+* serotype. No *ayr*, *adrq-* or *adw4* (*q+* or *q-*) serotypes were detected in the sample set (figure 5.3).

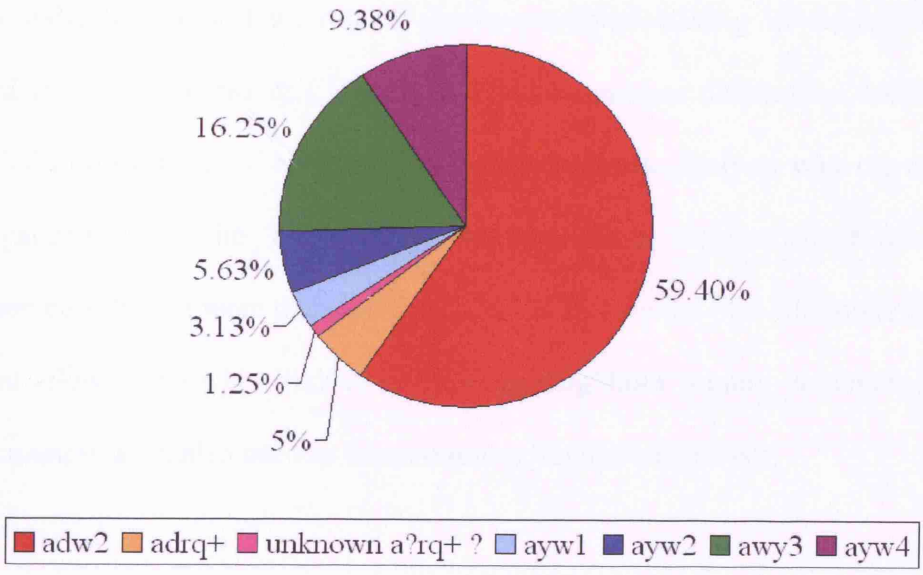


Figure 5.3 – Prevalence of predicted HBV serotypes present in sample set.

5.7: Preliminary phylogenetic analysis

It has been previously demonstrated that phylogenies derived from the entire S gene are congruent with those obtained from whole genome analysis (Kato *et al.,* 2002). The alignments generated from MEGALIGN (Lasergene, DNASTAR 1997) were exported to BIOEDIT (Hall 1999) upon which they were realigned using Clustal W (Thompson *et al.,* 1994) and converted to PHYLIP (Felsenstein, 1993) in a file format. Preliminary phylogenetic analysis was undertaken using the PUZZLE program (Strimmer & von Haeseler, 1996).

The overall transition/transversion ratio (Ts/Tv) as calculated by the PUZZLE for the 160 sequences, each 600 bp long, was = 2.10. The empirical estimate of base frequency as determined by PUZZLE was A: 19.8%, C: 27.8%, G: 21%, and T: 31.4%. The distribution parameter of invariant sites – α, was estimated by PUZZLE as $\alpha = 0.25$, which is indicative of a high rate of heterogeneity occurring in conjunction with conserved sites (McCormak & Clewley, 2002). The gamma distribution coefficient to be included in all models of evolution in the phylogenetic analysis was $1/\alpha$ or 1/0.25, and so gamma = 4. The Ts/Tv ratio, empirical base frequency and the gamma distribution co-efficient were then to be used in the F84 model of evolution (Felsenstein 1984) that allows for such variables in building neighbour joining phylogenetic trees; these parameters were also used in the maximum likelihood analysis.

5.8: Neighbour-Joining phylogenetic analysis

A distance matrix of the 160 sequences incorporating the parameters as determined by PUZZLE was prepared in DNADIST using the F84 model of evolution; this matrix was then used to prepare a phylogenetic tree in the program NEIGHBOUR for graphic representation by TREEVIEW (Page, 1996) [figure 5.4].

5.9: Maximum likelihood phylogenetic analysis

The maximum likelihood tree was prepared by entering the preliminary phylogenetic data calculated from PUZZLE and the 160-bp sequence alignment with the DNAML program into the PHYLIP suite of programmes. The calculated tree was presented graphically in TREVIEW (figure 5.5).

5.10: Bootstrap analysis of phylogeny

Bootstrap analysis of the sequences was performed in the program SEQBOOT within the PHYLIP suite of programs using 1000 replicates. The replicate figures obtained were then used to annotate the maximum likelihood tree that had been previously generated (figure 5.6).

5.11: Preliminary genotype analysis

The results from neighbour joining and maximum likelihood analysis were congruent (figures 5.5 and 5.6) indicating that the calculated phylogeny was likely representative of the actual phylogeny. No novel clades were identified, i.e., there were no untypeable sub-types, genotypes or potential trans-genotypic recombinants in the sample set. Moreover, the majority of the major clade branches were well supported by the bootstrap analysis. The proportion of genotypes observed is in figure 5.7.

For genotype A, a large proportion of variants observed were homogenous, 36/160 (22.5%) of the total sequences and 36/99 (36.4%) of genotype A sequences being identical. BLAST analysis and comparison to SBVL HBV sequence databases identified this variant to be HBV^{PV}, previously described to circulate commonly among acute hepatitis B in England and Wales (Hallett et al., 2004).

Five genotype C sequences were identical. Two separate groups of genotype D sequences were identical, one consisting of 8 variants and the other of 4 variants. Two sets of identical genotype E variants could also be identified, one consisting of 3 variants and the other of two variants.

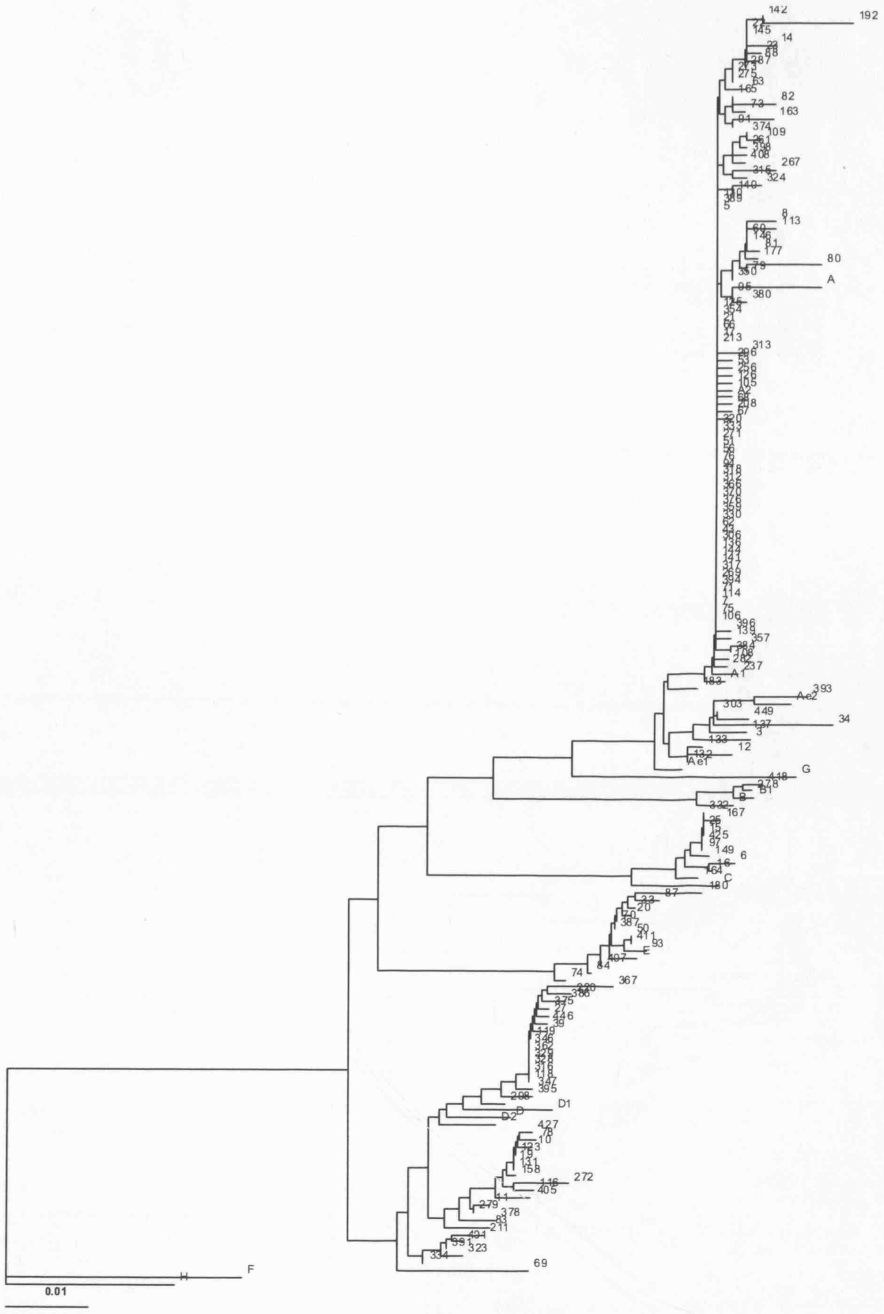


Figure 5.4 – Raw Neighbour-Joining analysis of 160 HBV variants, based on 1st 600 bases of S gene.

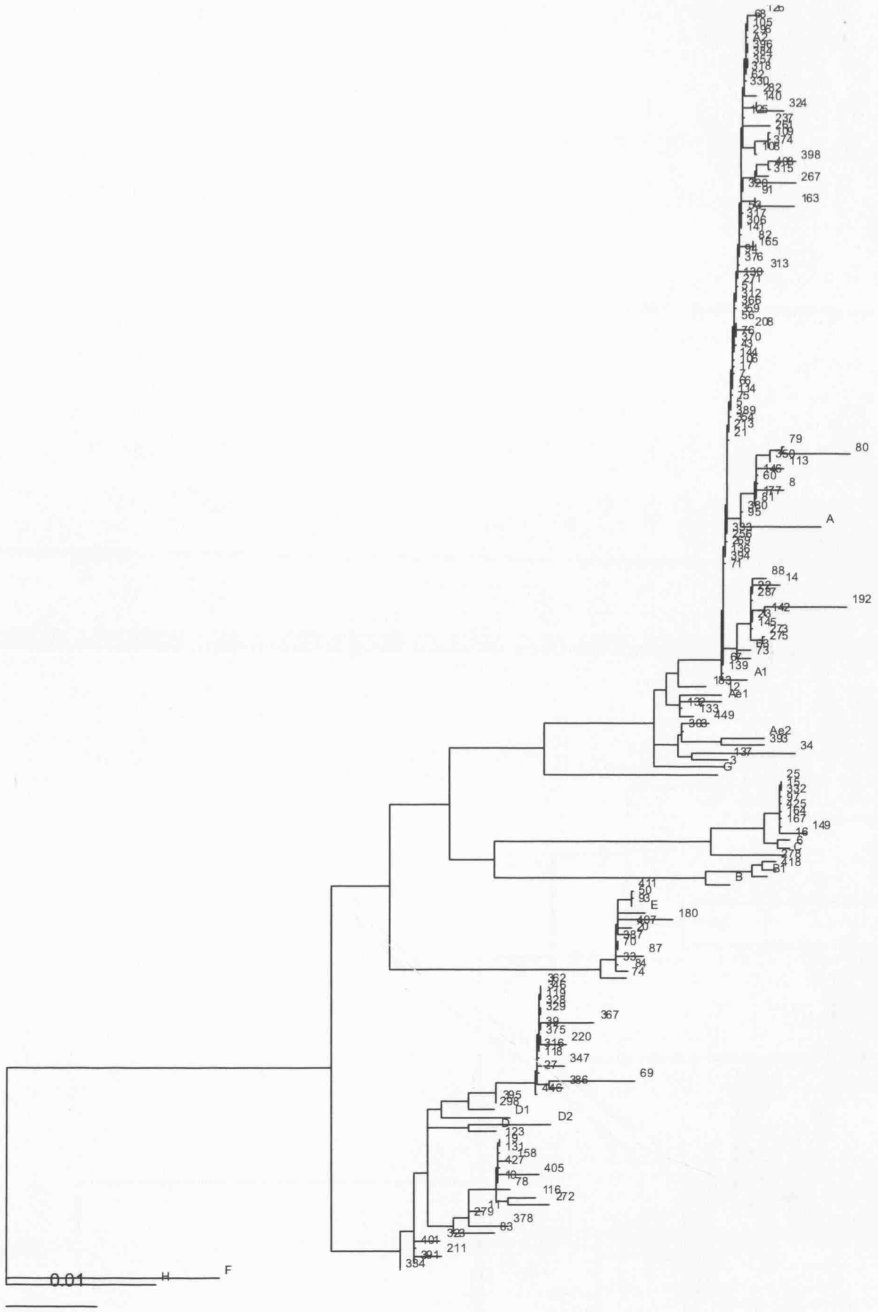


Figure 5.5 – Maximum likelihood analysis of 160 HBV variants, based on 1st 600 bases of S gene.

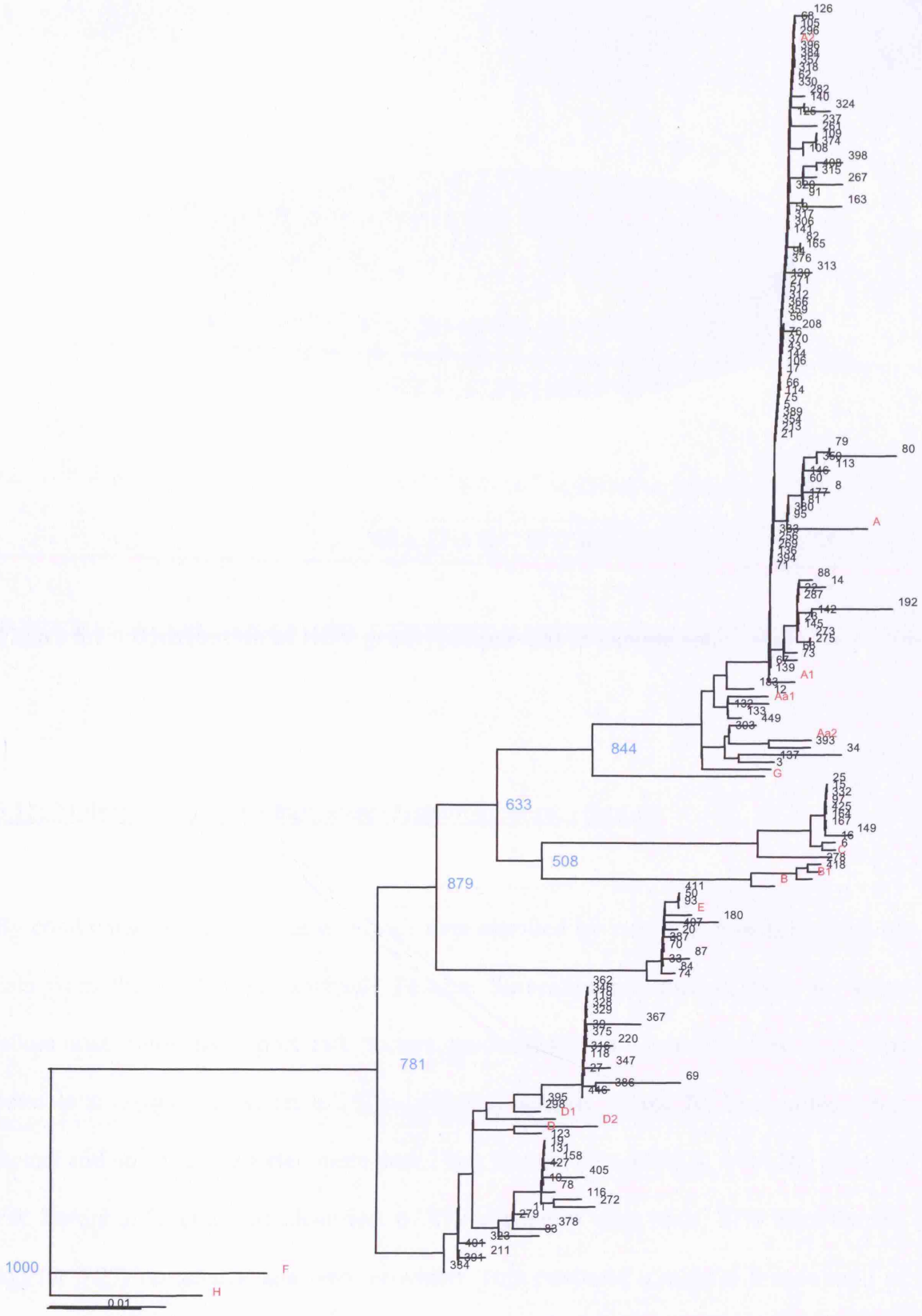


Figure 5.6 – Bootstrap analysis (1000 replicates) of maximum likelihood tree for the 160 HBV S gene variants.

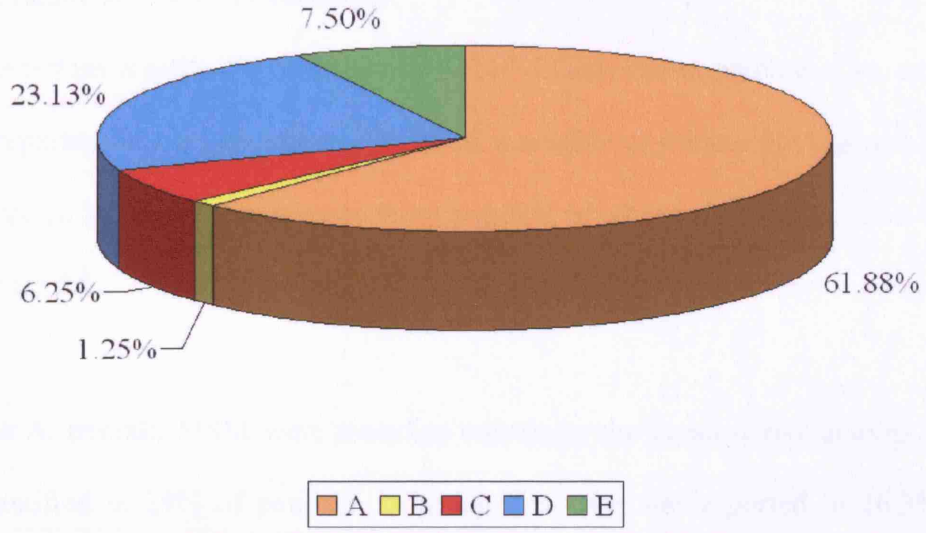


Figure 5.7 – Distribution of HBV genotypes present in sample set.

5.12: Molecular epidemiological-epidemiological correlation

By combining the sequencing data with data supplied by referring clinicians and with data from the HPA Communicable Disease Surveillance Centre (CDSC) to which laboratories routinely report risk factors associated with acute hepatitis B, it was possible to assign risk factors to 70/160 (43.8%) patients. Of the 70, 13 reported 2 risk factors and no patient reported more than 2 risk factors. Accordingly, from 160 patients, risk factors in 83 could be identified. 67.8% of patients were male, 27% were female, and for 5.2% no gender data were provided. This produced a male to female ratio of 2.51:1, consistent with that found in a recent HPA-based epidemiological study (Hahne *et al.,* 2004).

5.13: Molecular epidemiological analysis

5.13.1: Risk factor Analysis by Genotype

In order to ascertain whether there were noticeable differences in parenteral vs. sexual risk factors reported for each genotype identified, a neighbour joining phylogenetic tree was reconstructed using only sequences from patients in whom risk factors have been assigned (figure 5.8)

For genotype A, overall, MSM were found to constitute the greatest risk activity, and could be identified in 29% of patients. Injecting drug use was reported in 26.3% of patients.

Genotype A

Within genotype A 36/99 (36.4%) of all variants belonged to HBV^{PV}. Within all genotypes, HBV^{PV} was the most commonly observed variant, associated with 36/160 (22.5%) infections. Risk factors were available for 11/36 (30.6%) of the patients infected with HBV^{PV}. Of these 3/11 (27.3%) reported having travelled to or lived in an endemic country, 2/11 (18.2%) reported injecting drug use, and 4/11 (36.6%) men who have sex with men. Two patients infected with HBV^{PV} reported multiple risk factors: for one patient (male) reported these risks as transfusion and having sex with men, and the other patient reported injecting drug use and being in prison. Despite the finding that HBV^{PV} was the most often observed variant, the study sample was too small to allow definitive conclusions about the association between infection with HBV^{PV} and risk activities.

Nine patients were found to be infected with subtype Aa (Sugauchi *et al.*, 2004) which is a variant that is associated with endemicity in sub-Saharan Africa. Of the 9, in 3 risk factors could be found: one was known to have travelled to and lived in east Africa. Of the other two patients harbouring sub-type Aa virus who had risk factors available, one reported a history of travel or life in an endemic area (though no information as to country was available), the other reported a heterosexual contact.

Genotype B

Only 2 patients were found to be infected with genotype B. Of these 2, only 1 reported risk factors, which was having travelled to or lived in the Far East.

Genotype C

Four identical sequences were observed within genotype C. No epidemiological connection could be made between the four patients, although all reported having travelled to or lived in an endemic country and 2 also reported heterosexual contact as risk factor.

Two further genotype C-infected patients bearing non-identical virus reported risk factors, both of whom had travelled to or lived in an endemic country, one of whom reported heterosexual contact.

Genotype D

Genotype D was the second largest genotype seen in the study. Twenty patients reported risk factors. However, overall numbers reported for each risk group were too low to detect trends, as the largest number of patients in any risk group was 4 (who reported having travelled to or lived in an endemic country).

Infection in another 4 people by an identical variant was associated with 'health care exposure' as risk. These 4 patients could be identified to be patients involved in a large HBV outbreak due to alternative medical practices in London between 1997 to 1998 (Webster *et al.* 2000). Comparison of their HBV sequences to those held on the SBVL database confirmed that the sequences in them were identical to those identified for that outbreak.

A second group of 8 patients were found infected by one genotype D HBV variant. Only one patient in that group reported a risk factor (having travelled to or lived in an endemic area). There were no other epidemiological data suggesting a link between the patients.

Genotype E

Risk factors were only available for 5 of the 12 genotype E-infected patients. No identical variants were observed, and no clear pattern of infections within risk factor groups could be inferred.

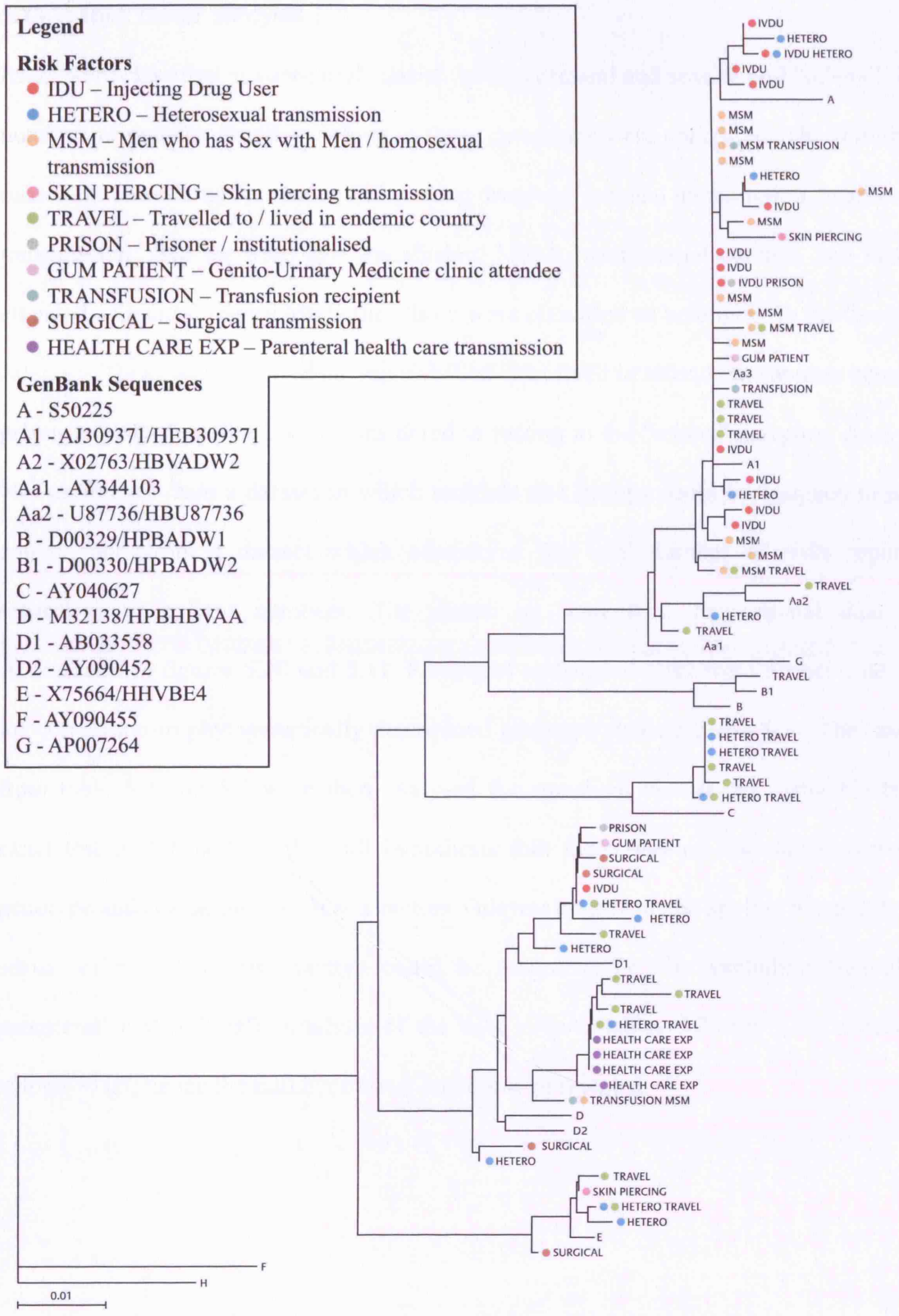


Figure 5.8 – Neighbour-Joining phylogenetic tree based on 1st 600 bases of HBV surface gene. Constructed only from patient sequences for which risk factor was available.

5.13.2: Risk factor analysis

Risks were classified as parenteral, sexual, both parenteral and sexual, and "others". The numbers of patients found to belong to these categories were compared. The following risks were considered "parenteral": injecting drug use, surgical transmission, health care transmission, skin piercing, and transfusion. MSM, heterosexual contact, and having attended a Genito-Urinary Medicine Clinic were classified as belonging to the "sexual" category. Those who reported having travelled to or lived in an endemic area or being in prison or institutionalised were considered to belong to the "others" category. Analysis was performed from a dataset in which multiple risk groups could be assigned to each patient, and from a dataset which considered the total number of risks reported regardless of patient numbers. The sexual vs. parenteral proportional data are summarised in figures 5.10 and 5.11. Parenteral and sexual risks were further analysed for correlation to phylogenetically determined genotype (table 5.2 and 5.3). The results from table 5.2 and 5.3 were then analysed for statistical correlation using Fischer's exact test to substantiate the null hypothesis that there was no correlation between genotype and sexual/parenteral risk factors This test could only be applied when definite sexual or parenteral risk factors could be assigned (i.e., by excluding 'sexual + parenteral' and 'others'). Analysis of the data in both tables 5.2 and 5.3 produced p values >0.05; hence the null hypothesis could not be rejected.

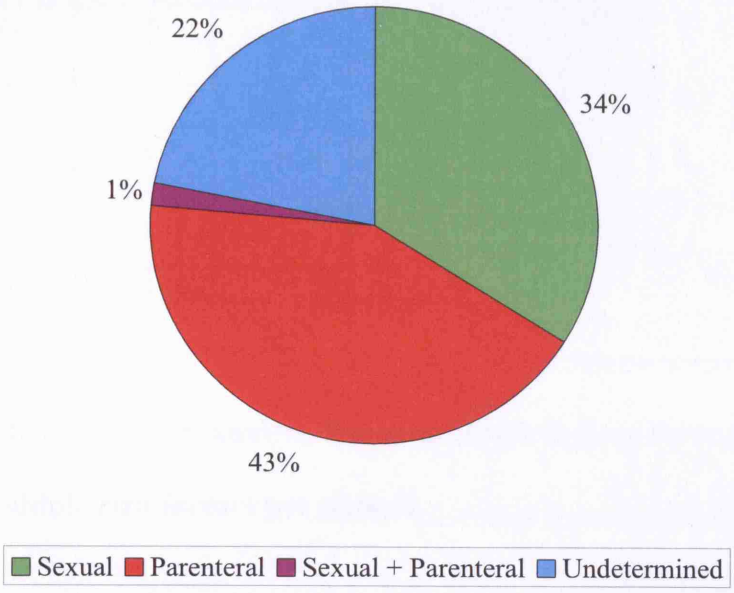


Figure 5.10 – Proportion of Sexual and Parenteral risk factors allowing for multiple risk factors per patient.

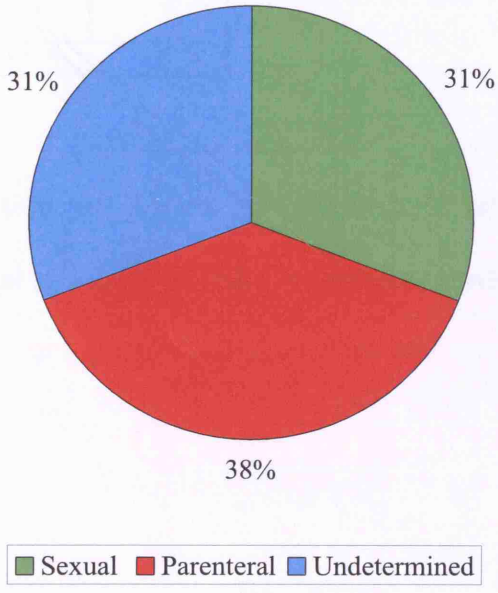


Figure 5.11 – Proportion of total reported Sexual and Parenteral risk factors regardless of patient numbers.

	Risk Factor		
Genotype	**Sexual**	**Parenteral**	**Total**
A	22	18	40
B			
C	3		3
D	7	5	12
E	2	2	4
F			
G			
H			
Total	34 (57.6%)	25 (42.4%)	59

Table 5.2 – Distribution of Sexual vs. Parenteral risk factors throughout genotypes allowing for multiple risk factors per patient.

	Risk Factor		
Genotype	**Sexual**	**Parenteral**	**Total**
A	15	22	37
B			
C	3		3
D	9	7	16
E	2	2	4
F			
G			
H			
Total	29 (48.3)	31 (51.7%)	60

Table 5.3 – Distribution of Sexual vs. Parenteral risk factors throughout genotypes, based on total numbers of risks reported regardless of patient numbers.

5.13.3: Correlation between HBV genotype with predicted HBsAg serotype

Risk factor analysis according to predicted HBsAg serotype. The neighbour joining tree was annotated with the predicted serotypes to demonstrate their distribution throughout the genotypes (figure 5.9). The correlation between the 2 was not always close, an observation already noted by others (Okamato *et al.,* 1988; Norder *et al.,* 1992; Norder *et al.,* 1993; Magnius & Norder, 1995; Norder *et al.,* 2004). Genotype was tabulated against predicted serotype to document the relationship between the two. This dataset is summarised in table 5.4 below. The null hypothesis that there was no correlation between genotype and predicted serotype was investigated. While a chi square analysis would be applied to such a dataset, the numbers in some of the categories were very low (i.e. < 5). Fischer's exact test was then considered; however, the large number of categories (due to the number of predicted serotype groups) made this difficult. Hence, a second table was drawn, segregating the predicted serotypes into 2 groups: those with predicted *ad* and those with predicted *ay* specificity (table 5.5). When Fischer's exact test was applied to this dataset, the null hypothesis being that there was no correlation between predicted *ad/ay* serotype and genotype factor, the *p* value obtained was <0.001. Hence the null hypothesis could be rejected.

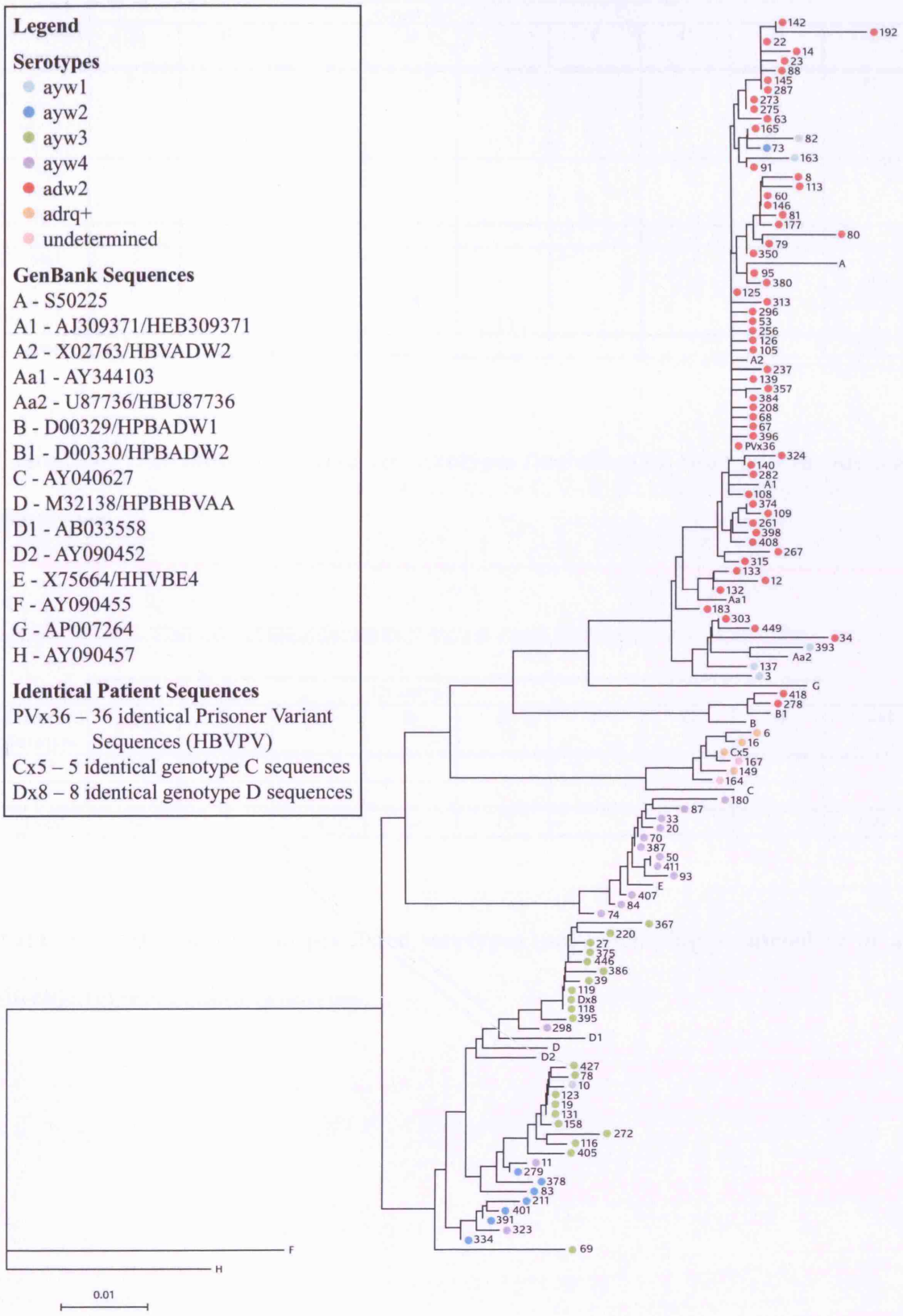


Figure 5.9 – Neighbour-Joining phylogenetic tree based on 1st 600 bases of HBV surface gene, annotated with predicted serotype.

	Genotype								
Predicted Serotype	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**Total**
adw2 *adw4* *adw4q+* *adw4q-*	93	2							95
adrq+ *adrq-* *aqr*			8						8
ayr									
ayw1	5			1					6
ayw2	1			7					8
ayw3				28					28
ayw4				3	12				15
Total	99	2	10	39	12				162

Table 5.4 – Distribution of predicted serotypes (including all subtypes) throughout genotypes.

	Genotype								
Predicted Serotype	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**Total**
ad	93	2	8						103
ay	6			39	12				57
Total	99	2	8	39	12				160

Table 5.5 – Distribution of predicted serotypes (only including predicted *ad* or *ay* specificity) throughout genotypes.

5.13.4: Risk factor analysis by predicted serotype

As for genotype, patient risk factors were segregated into the sexual, parenteral, sexual + parenteral, and "others" categories. The data are presented in tables 5.6 and 5.7. Fischer's exact test was used applied to examine the null hypothesis that there is no correlation between predicted subtype and sexual/parenteral risks. The *p* value was > 0.05; hence the null hypothesis could not be rejected.

This analysis was then repeated, though grouping predicted serotypes into either predicted *ad* or *ay* specificity (table 5.8 and table 5.9 below). No statistically significant differences could be found ($p > 0.5$).

	Risk Factor		
Predicted Serotype	**Sexual**	**Parenteral**	**Total**
adw2 *adw4* *adw4q+* *adw4q-*	22	18	40
adrq+ *adrq-*	2		2
aqr			
ayr			
ayw1		1	1
ayw2	2	1	3
ayw3	3	4	7
ayw4	3	1	4
Total	33 (56.8%)	25 (43.1%)	58

Table 5.6 – Distribution of Sexual vs. Parenteral risk factors for all predicted serotypes allowing for multiple risk factors per patient.

	Risk Factor		
Predicted Serotype	**Sexual**	**Parenteral**	**Total**
adw2 *adw4* *adw4q+* *adw4q-*	25	21	46
adrq+ *adrq-*	2		2
aqr			
ayr			
ayw1		1	1
ayw2	3	1	4
ayw3	4	5	9
ayw4	4	2	6
Total	39 (56.5%)	30 (43.5%)	69

Table 5.7 – Distribution of Sexual vs. Parenteral risk factors for all predicted serotypes, based on total numbers of risks reported regardless of patient numbers.

	Risk Factor		
Predicted Serotype	**Sexual**	**Parenteral**	**Total**
ad	24	18	42
ay	8	7	15
Total	32	25	57

Table 5.8 – Distribution of Sexual vs. Parenteral risk factors for predicted serotypes (only including predicted *ad* or *ay* specificity) allowing for multiple risk factors per patient.

	Risk Factor		
Predicted Serotype	**Sexual**	**Parenteral**	**Total**
ad	27	21	48
ay	11	9	20
Total	38	30	68

Table 5.9 – Distribution of Sexual vs. Parenteral risk factors for predicted serotypes (only including predicted *ad* or *ay* specificity) , based on total numbers of risks reported regardless of patient numbers.

5.14: Discussion

No *pol* or S gene mutants that could be attributed to being iatrogenically induced were identified in this study. There are many reasons, both virological and epidemiological, to account for this paucity.

It has been considered that HBV in an infected individual exists as quasispecies (Smith *et al.,* 1997). The proportions of individuals within the quasispecies are determined by the selective pressures placed upon them, which may be immunological or imposed by an antiviral drug. In this study DNA sequences were generated from PCR amplicons by consensus sequencing, and so the consensus sequence obtained would be representative of the dominant strains in the quasispecies. Minority strains are only detectable by consensus sequencing when they are present above a proportion of *ca.* 25%. If higher than 25%, the sequence motifs of the minority strains become visible chromatographically (Punia *et al.,* 2004). If an iatrogenically induced mutant were to be present within an individual, it is possible that it may exist as part of quasispecies and represent a minority constituting <¼ of the total population. As lamivudine-resistant HBV mutants are particularly associated with a reduced replicative capacity (Melegari *et a,,.* 1998), such mutants may be present in the patients studied but remain undetectable. In such circumstances, quasispecies mutants may be detected by clonal analysis of multiple clones derived from PCR amplicons. Furthermore, once the resistant virus has been transmitted to a newly infected individual, the selection pressure of the drug would be removed and the resistant virus (of lower replicative capacity than wild type) would revert to the more replication-fit wild type genome. This phenomenon has been observed in patients from whom lamivudine therapy has been withdrawn after

a substantial period of treatment (Buti *et al.,* 1997; Chayama *et al.*, 1998; Lau *et al.* 2000; Da Silva *et al.*, 2001).

The scarcity of HBV *pol* gene mutants in the sample set may also be explained by the low transmission rate of the mutants. Transmission of HBV antiviral resistance mutants has only been documented once (Thibault *et al.,* 2003). In this instance, the patient was initially seronegative for hepatitis B markers, but was anti-HIV-seropositive and receiving HAART which included lamivudine. Upon admission to hospital with clinical hepatitis, primary HBV infection was serologically diagnosed and the infecting HBV variant identified as bearing the rtM204V/sI195M+rtL180M mutations. Lamivudine was maintained and in spite of this, serum HBV DNA levels rose, eventually subsiding when therapy was removed. Transmission of the mutant must have been from a patient who was HBV-infected, possibly co-infected with HIV, and who was receiving lamivudine. Transmission under such circumstances would be expected to occur rarely, given that the lifetime risk of chronic infection with HBV in the UK is 0.04% (Hahne *et al.,* 2004). Moreover, not all chronically infected people, who potentially constitute a reservoir, would be receiving antiviral therapy, and an even smaller subset would develop resistance. Furthermore, transmission would only occur if this subset of patients with resistant virus infects those taking the same drugs (either against HIV or HBV).

Not all HIV or HBV infected patients may be on the same HAART or antiviral regime due to the increasing number of licensed antivirals against both HBV and HIV. Already, adefovir, and more recently entecavir have been given licensure for the treatment of chronic HBV infection. Adefovir drug is associated with a resistance rate of 5.9% over 144 weeks (Hadziyannis *et al.,* 2005) and is effective against lamivudine-resistant mutants (Xiong *et al.*, 1998; Ono-Nita *et al.,* 1999; Perrio *et al.,* 2000, Benhamou *et al.,*

2001). Even more recent is the licensure of entecavir, to which lamivudine-resistant mutants show partial cross resistance (Yang *et al.,* 2005). However, drugs such as tenofovir and emtriltabine, which are in Phase III clinical trials and are effective against HIV and HBV (Peters, 2005), may also have the potential to enhance the transmission of HBV drug-resistant mutants as the pool of susceptible patients is not just restricted to HBV-infected patients on therapy, but includes those on HAART in which such drugs are incorporated. However, new ranges of HBV specific drugs, the β-L-nucleosides, including telbivudine, which is in Phase II development, are HBV- but not HIV-specific and so are not prone to infect HAART patients. Although the number of HIV infected patients in the UK receiving HAART is increasing (Rice *et al.,* 2005), the overall risk of chronic infection in the UK is low, and the HIV prevalence in the UK is also relatively low (Rice *et al.,* 2005), so the likelihood that such mutants will emerge within the UK population would be correspondingly low.

YMDD mutants may pre-exist in some chronically infected patients who are not yet treated with lamivudine. Thus, mutants were detected at the rate of 5/18 patients using PCR-ELISA and PCR-enzyme-linked mini-sequencing [ELMA] (Kobayashi *et al.*, 2001), 8/36 patients using peptide nucleic acid amplification (PNA), (Kirishima *et al.*, 2002), 3/40 patients using oligonucleotide chips in conjunction with RFLP (Heo *et al.*, 2004) and 1/3 patients using Line Probe Assay [LiPA] (Leon *et al.*, 2004). These studies have all relied on non-sequencing or mini-sequencing-based techniques. No sequencing was performed and so it is unknown in what genetic backbone and context such mutations lay. Only one study to date claims to have detected YMDD mutants using direct sequencing, and 3/40 patients were found to carry the mutants (Shin *et al.*, 2003). Efforts to replicate such work using a combination of PCR, RFLP and SMITEST (a commercially available SNP assay) failed to replicate results in 20 untreated chronically

infected patients (Matsuda *et al.,* 2004). Another study which observed the rapid emergence of lamivudine resistance in multiple patients sought to explain this observation in terms of pre-existing lamivudine-resistant mutants having arisen from within chronic carriers in an endemic population [South Korea] (Paik *et al.,* 2001). Only in one instance was a lamivudine-resistant mutant detected as primary infection (Thibault *et al.,* 2003).

The PNA assay as used by Kirishima *et al.* 2002 is known to be extremely sensitive, capable of detecting 0.01-0.001% of mutant viruses coexisting in 10^5-10^9 copies of wild-type viruses. YMDD mutants may be viable, although less 'fit' than their wild type counterparts (Melegari *et al.*, 1998), so it is possible to consider that such mutants may appear as a minor species in the quasispecies, but have not been positively selected in the absence of antivirals. As little is known about the surrounding sequence of the YMDD mutants detected in this study other than specifically at the YMDD motif, it is thus unclear such virus was viable. Nonetheless, it may be possible that complementation – the situation whereby the presence of a mutant gene copy is compensated for by the presence of a viable virus within the same cell – may be occurring (Delaney *et al.*, 2001).

A study from Spain which reported lamivudine-resistant mutants in untreated chronically infected patients have not explained the data in terms of resistant mutants arising within the individual, but in terms of transmission of lamivudine-resistant variants within the population (Leon *et al.*, 2004). However, in Spain, lamivudine was licensed for the treatment of chronic hepatitis B only in 2001, and that study was carried out in patients screened between January 2001 and May 2002. Given a lamivudine resistance rate of 14% over 1 year and 38% over 2 years (Leung *et al.,* 2001; Liaw *et*

al., 1999), there seems insufficient time for the degree of transmission of lamivudine-resistant mutants (4% of all Spanish chronic carriers) to be reached. That study should have focused on incident infections, and examined for YMDD mutants present as minority strains.

From a general point of view, if lamivudine resistant variants are indeed emerging from untreated chronically infected patients, the pool of patients potentially able to transmit mutant virus may be larger than previously considered. Virus may be transmitted to a patient and remain in the minor population until selective pressure is exerted when a host is given lamivudine therapy (Paik *et al.,* 2001).

In infants born to HBeAg mothers who receive inefficient passive-active vaccination due, e.g., to delay in administration, the likelihood of selecting S gene mutants increases (Carman *et al.,* 1990). Such mutants can also be transmitted vertically. As most transmission in the UK is horizontal and not vertical, the opportunities for vaccine escape mutants to emerge due to post-natal immunisation practices in infants are less, particularly if efficient antenatal screening of infants occurs (NHS 1998; Salisbury and Begg 1996).

The absence of S gene 'vaccine escape' mutants may also be associated with the hepatitis B selective immunisation policy currently implemented in the UK, and also to poor coverage across all risk groups (Hahne *et al.,* 2004). Given this low rate of immunisation, there may be insufficient selective pressure for the emergence of vaccine-escape mutants. Modelling studies suggest that it would take 30 years of universal immunisation for vaccine resistant mutants to become dominant (Wilson *et al.,* 1998; Wilson *et al.* 2000). However, this analysis was based on a study in Taiwan,

which implemented a universal vaccination program in 1984. In view of the selective vaccination policy in the UK, the selection pressure that would generate vaccine-escape is likely to be much weaker and their emergence within the acutely infected population less likely.

Work described in other chapters of this thesis highlight that at the nucleotide level, mutations selected for either vaccine/HBIG escape or antiviral resistance can be identical despite the source of the selective pressure being different. It has been demonstrated that the lamivudine-resistant mutant rtM204V/sI195M+rtV173L can be as unreactive in certain immunoassays as the classical G145R vaccine escape mutation (Torresi *et al.,* 2002b). Moreover, it has been shown that the typical vaccine escape mutant G145R can be generated to compensate for the replication-unfit state of lamivudine mutants (Torresi *et al.,* 2002a). Thus, antiviral resistance mutations can lead to vaccine escape and vice versa. Consequently, we must consider that vaccine escape and antiviral resistance can in certain circumstances be one and the same; selection for one form of iatrogenic mutation (i.e. drug resistance or vaccine escape) can effectively lead to another form of iatrogenic mutation (i.e. vaccine escape or drug resistance). This should be considered when calculating the potential for iatrogenic mutants to emerge as acute incident HBV infections within the UK population. The issue of one form of iatrogenic resistance leading to another has left some researchers observing mutations that they were unable to explain if they have been selected as a consequence of antiviral or anti-HBs therapy (Lee *et al.,* 2005).

Despite this consideration, there is no strong hypothesis for expecting to observe vaccine resistant or antiviral resistant mutants in the UK population in the period studied (1997-2001) due to low incidence and the lack of universal vaccination policy.

Moreover, once a resistant mutant has been selected, transmission must occur between individuals whose virus population is concurrently under the same selective pressure (or wild type reversion will occur). The incidence of HBV infection is low. The likelihood that transmission to a second individual who is on identical therapy would be even rarer.

However, it is possible that resistant mutant virus may be selected as a minority proportion of quasispecies population in an untreated chronically infected patient. Although it is still unclear if this actually occurs *in vivo* as data from the work of Kobayashi *et al*, (2001), Kirishima *et al.* (2002), Shin *et al.* (2003), Heo *et al.* (2004) are in disagreement with those of Matsuda *et al.* (2004), if true such emergence may constitute another source of resistant virus entering the population. Thus in future when calculating the potential incidence of resistant mutant virus within the population, resistant mutations arising as a consequence of mutational change in an overlapping gene, or emergence as a minority population in a patient quasispecies population must be considered. This consideration may affect the surveillance of resistant mutant virus entering the HBV-susceptible population.

In the course of correlating epidemiological data to those generated from phylogenetic analysis, a problem with regard to the definition of incident HBV infection was encountered. A cut-off of 200 PEI/ml anti-core IgM was set, and – indeed - patients undergoing acute resolving infection will have high anti-core IgM titres within this range compared to chronically infected patients (Tedder *et al.*, 1981; Hollinger & Dienstag, 1995). However, chronically infected patients undergoing hepatic flares also produce high anti-core IgM titres (Mels *et al.,* 1994; Perillo *et al.,* 2001). This phenomenon has also been observed in HBeAg-positive infected patients in whom high-replication chronic infection occurs (Liaw *et al.,* 1985; Mels *et al.,* 1994; Perillo *et al.,*

2001) and in HBeAg-seropositive children (Bortollotti *et al.,* 1990) as well as those in the HBeAg-seronegative chronic state (Perillo *et al.,* 2001). It is likely that sera from such patients would also have been included in the analysis. This is perhaps indicated through the median age of patients in this study, which was 43.5 years of age, higher than the 36 years of age observed in a much larger study (Hahne *et al.*, 2004). Thus it is possible that this figure indicates that patients chronically infected with HBV have been included in this study (as they would tend to be older). Though it must be noted that overall patient numbers in this study were up to twenty times lower than that of Hahne *et al.,* 2004 and the two sets of data are not directly comparable.

Knowledge of viral genotypic distribution in a population is becoming increasingly useful, especially when considering the increasing body of evidence in favour of the notion that HBV genotype significantly influences disease outcome (Wai & Fontana 2004; Schafer, 2005). Although such genotype correlations are not comprehensive, especially when considering the common European genotypes (A & D), genotypes are now known to influence likelihood of progression to chronic infection (Kikuchi *et al.*, 2000), severity of chronic disease (Kao *et al.*, 2000), likelihood of HCC (Tsubota *et al.*, 2001), appearance of HBeAg-negative chronic infection (Orito *et al.*, 2001), response to vaccine (Milich *et al.* 1990a; Milich *et al.,* 1990b), likelihood of vaccine escape mutants being selected (Ngui *et al.,* 1998), response to interferon therapy (Kao *et al.,* 2000; Wai *et al.,* 2002; Janssen *et al.,* 2005), rate at which antiviral resistance emerges (Buti *et al.,* 2002), and type of resistance-associated mutation under lamivudine therapy that may develop (Hadziyannis *et al.,* 2000). No formal study of the proportion of HBV genotypes within the English population has been reported. This is despite similar reports from Asia (Thakur *et al.,* 2002), Africa (Borchni-Chabchoub *et al.,* 2000), North America (Chu *et al.,* 2003), Australia (Alestig *et al.,* 2001) and Europe in countries such

as Spain (Echevarria *et al.,* 2005), Poland (Bielawski *et al.,* 2004) and the Netherlands (van Steenbergen *et al.,* 2002) having been undertaken. In Scotland, the distribution of genotypes has been observed to be 41% for genotype A, 12% for genotype B, 5% for genotype C, 30% for genotype D, and 12% for mixed-genotype infections (Davidson *et al.,* 2005).

Analysis of major branches in the maximum likelihood tree completely supported the neighbour joining phylogeny demonstrating the robustness of the tree produced. All significant clades (genotype splits) were supported by replicates of over 700/1000 (McCormack & Clewley, 2002). Findings from this dataset are concordant with phylogenies based on similar sequence lengths (Norder *et al.,* 2004). Given this confidence it was then possible to ascertain genotype.

No variants were identified in this study which did not cluster with already known genotypes, and no heterotypic (mixed genotypes) infections were identified. The lack of mixed genotypes does not reflect the true rate of super-infection with mixed genotypes, - other studies have reported having detected double infections at rates from 4.4% to 14.1% (Schaefer *et al.,* 2005) - but such paucity is a reflection on the genotyping technique used, since consensus sequencing of PCR is unsuitable for accurately defining mixed infection.

Genotype A and D were found predominant in the study samples, an outcome expected for a northern European or North American population (Norder *et al.,* 2004). A substantial proportion of the samples - 1.25%, 6.25% and 7.5% - were found to carry genotypes B, C and E, respectively, which represents the genotypes from East Asia (B and C) and Africa (E) (Norder *et al.* 2004) The recently identified genotype A subtype,

Aa, which is typically found in sub-Saharan Africa (Sugauchi *et al.,* 2004) was also present in the study population at a rate of 5.6%, and accounted for 9.1% of all genotype A infections. In aggregate, these 'exotic' genotypes and subtypes represented 20.6% of all infections in this study. Immigration is the likely source of 'exotic' genotypes within the British population, although this notion cannot be formally confirmed in this study owing to the poor return of patient epidemiological data, particularly in regard to the country of origin. Other epidemiological studies in England and Wales have documented immigration as an important factor in the epidemiology of HBV in the UK (Hahne *et al.,* 2002). Another study, based in the Netherlands, has also suggested that immigration is responsible for the genotype distribution observed in Western European populations (van Steenbergen *et al.,* 2002).

The proportion of all predicted serotypes was determined from the same dataset. Analysis to correlate genotypes with serotypes showed concordance with data observed in other studies (Norder *et al.,* 2004). However, serotype is increasingly less used in epidemiological studies and may be of little value in its own right, although there has been suggestion that serological subtype may influence response to vaccination (Milich *et al.,* 1990a; Milich *et al.*, 1990b).

Two variants were identified in which serotype could not be predicted from the small S gene sequence. They possessed a predicted glycine residue at surface codon s122, so the *d/y* type could not be determined. Analysis of the remainder of the predicted aa sequence for these two variants revealed a predicted *rq+* specificity. Phylogenetic analysis showed them to belong to genotype C and to cluster amongst *adrq+* serotypes (all of which were exclusively genotype C). The variants were not identical, nor was

there any epidemiological linkage between them, although both patients indicated having travelled to or lived in an endemic country.

Variants with neither *d* nor *y* determinant have previously been reported (Echeverria *et al.,* 2005). It is unclear what the impact would be, in terms of antigenicity, upon the glycine substitutions at the codon responsible for predicting *d/y* antigenic specificity, as was observed in two variants in this study. Arginine (R/ *d*) and lysine (K/*y*) are both positively charged, polar, basic aas, whereas glycine though also is polar is not positively charged. Thus, in this substitution, positive charge is lost. It is likely that these variants are mutants of *adrq*+/genotype C variants which have substituted glutamine at codon 122 for glycine, which although not basic is still fairly conservative and so may be structurally tolerated by the virus despite the loss of *d/y* determinants. Sequence analysis could not determine the *q* determinant for these two variants as both codons (s159 and s177) involved in *q* subtype determination had characteristics of *q*+ and *q*- subtypes. It is unknown how this variant would behave in a true serological assay. As consensus sequencing was used, it is possible that this mix of *q* determinants is artefactual and is the result of both *q*+ and *q*- determinants being present in the sample.

Analysis of risk factors in the study population, known in 44% of the sample, identified IDU and MSM as the principal groups at risk of infection (15.7% of each group within the total study population). The dominance of these 2 risk groups agrees somewhat with that in the study by Hahne *et al* .(2004) which examined more patients (n = 1144); injecting drug use was found to be the greatest risk factor (53% in males and 43% in females) whilst male homosexual intercourse was implicated in 21% of male cases.

This study highlighted travel to or having lived in an endemic country as a major risk for 20% in patients with risks or 28% with all risks reported (this latter figure includes patients reporting multiple risks). However, the wording of this risk factor in the questionnaire makes it difficult to differentiate between immigration and travel. Furthermore, when not combined with any other risk, it is unclear whether the mode of transmission was parenteral or sexual. When transmission risk was provided with the travel risk, the data provided were more meaningful: 8.6% of patients with risk factors reporting the combined risk factors of heterosexual contact and 'travel to / lived in an endemic country'. However, it is unclear in this study to what extent this risk group carried HBV imported through immigration spread by heterosexual sex or by uninfected people travelling abroad and then acquiring infection. Hahne et al. (2004) showed that the net immigration of persons chronically infected with HBV was 6751 per year; hence, only 3.9% of chronic infections in England & Wales could be considered indigenous. That study also indicated that amongst South Asians acutely infected with HBV and with a history of travel to South Asia, medical treatment was the most common transmission risk.

It was also difficult to define parenteral or sexual transmission for the 'Prison' risk group as it is unclear if transmission arose from parenteral or sexual transmission, although it is suspected that the majority of transmission is through drug use rather than MSM sexual transmission (Gill *et al.,* 1995; Strang *et al.,* 1998). A high proportion of IDUs have a history of being in prison (Department of Health 2000), and universal immunisation of prisoners against HBV has consequently been suggested as a means to control infection within this population (Hutchinson *et al.,* 2004). For the genito-urinary-medicine (GUM) patient category, although sexual transmission is implied this is not necessarily the case, as 3.9% of male of GUM attendees report injecting drug use

or contact with an IDU (Gilson *et al.*, 1998) It has also been shown in the Netherlands that high risk sexual behaviour associated with drug use (such as prostitution) is the source of many secondary infections (van Steenbergen *et al.*, 2002).

One aim of this study was to investigate risk factors within genotypes, but such work was difficult due to the under reporting of risk factors by referring clinicians. There appears to be no clear distinct segregation of risk factors by genotype, unlike the situation observed in a recent Dutch study (van Steenbergen *et al.*, 2002) in which all MSMs were found segregated into genotype A and all IDUs into genotype D. As such a large proportion of sequences were of genotype A (61.9%) analysis by genotype was difficult as the numbers in the other genotypes were small. Moreover, the small numbers of individuals identified per risk factor made analysis difficult; e.g., injecting drug use was one of the most commonly reported risk factors, yet within genotype A (the most commonly observed genotype), only 11 instances were seen.

The HBV 'prisoner variant' [HBV^{PV}] (Hallet *et al.*, 2004) within the study population was found to be prominent. HBV^{PV} was present in a similar proportion (36/99, 36.4%) of all genotype A sequences and 36/160 (22.5%) of all variants when compared with the previous work by Hallet et al. (2004) in the 1990-1996 time period which found HBV^{PV} to be present at a rate of 16-37%. The implication of the original HBV^{PV} work by Hallet et al. (2004) was that strains with a specific sequence pattern may spread from at-risk groups into the general population within a relatively short period (Zollner, 2004). This study demonstrates that HBV^{PV} has persisted and remained the major variant in southern England, confirming its transmissibility and stability. HBV^{PV} was associated with a broad range of risk factors including MSM, 'travel to or lived in endemic area', 'prison' and 'prior transfusion', implying that it is not restricted to just the

institutionalised and IDUs as was originally observed. There are no data provided in this study to help explain why this variant is so predominant.

The study described in this chapter highlights how surveillance of HBV variants can be an essential precursor in the implementation of public health programmes. As in the case of HIV, the awareness that there is a growing heterosexual epidemic in the UK that overshadows the epidemic in MSM which may require novel public health strategies to be in place (Rice *et al.,* 2005). The ability to track the spread of HBV^{PV}, for example, demonstrates the finesse that is potentially inherent to current molecular epidemiological surveillance. Ongoing surveillance to identify and track novel variants, whether iatrogenic or not, should continue to be implemented

CHAPTER 6

OVERALL DISCUSSION AND CONCLUSIONS

6.1: Introduction

The data presented in chapter 4 of this thesis show that the selection of antiviral resistance mutations, particularly in combination, can abrogate the major epitope for anti-HBs to bind to HBsAg. There are potentially serious implications which arise from these observations. In this final chapter, the data firstly will be considered at the nucleotide level (section 6.2). The constraint imposed by overlapping genes will be discussed. Next, the data will be discussed at the amino acid and protein level. The data obtained are then related to the currently proposed structural models for HBV and defined epitopes for anti-HBs (section 6.3). Thereafter the data will be placed in a virological and then epidemiological context, such as the selection of immunological escape mutants. This discussion will first draw on data obtained from chapter 5 and the possibility of selection of mutations such as those in both developed and developing counties will be contrasted (section 6.4). Next, it will be argued that such mutations have the potential to further subvert diagnostics and antiviral therapy (section 6.5). It must be also considered if the phenomena observed in chapter 4 of this thesis are likely to be unique to HBV (section 6.6). Finally, any future work that might improve understanding of the phenomena observed is proposed (section 6.7).

6.2: Antiviral mutations: implications on the nucleotide level

A unique feature of the HBV genome is that the S gene is entirely overlapped by the polymerase gene. The first nucleotide in a *polymerase* codon is the second of a *surface* codon (see figure 6.1 below). Despite replication via proof reading lacking reverse transcriptase the variety of mutations selected by HBV in the S gene overlapping *pol* is less than for retroviruses (Ngui *et al.*, 1990). This discrepancy is attributable to the genetic constraint of mutations being tolerable in both ORFs. Thus, when single nucleotide mutations are observed which affect both ORFs the impact must be considered in terms of overall selective advantage to the virus, not just the single gene.

Surface: F S Y M/V* D D

Nucleotide: T T C A G T T A T A/G* T G G A T G A T G T G

Nucleotide: T T C A G T T A T A/G* T G G A T G A T G T G

Polymerase: S V I/M* W M M

Figure 6.1: Overlapping *surface* and *polymerase* ORFs for HBV. The data is for wild type and the mutant rtM204V/sI195M.

It is relevant that the 1st nucleotide of each *pol* ORF acts as the second nucleotide in the S gene ORF. Due to the redundancy of the genetic code, non-synonymous changes in *pol* will most likely be in the first and second nucleotides of a codon. Thus, when single-nucleotide antiviral resistance mutations arise as a consequence of selection in *pol*, they are most likely to be reflected in changes in the 3rd and 1st codons of the S gene. Those that arise in the 3rd nucleotide of a surface codon are more likely to be synonymous than those that arise in the 1st nucleotide. Such occurrences are exemplified

by the lamivudine resistance compensatory mutation rtL180M/sSilent and the tenofovir resistance mutation rtA194T/sSilent (Sheldon *et al.*, 2005). These considerations will affect the frequency to which *pol*; selected antiviral resistance mutations will emerge in the S gene. Bearing this in mind, the site-directed mutagenesis in this study all involved single amino acid substitution that affect both ORFs. It has previously been observed that antiviral resistance mutations in HBV *pol* may influence the S gene (Tipples *et al.*, 1996; Bartholomew *et al.*, 1997; Terrault *et al.*, 1998; Ogura *et al.*, 1999; Shields *et al.*, 1999; Lok *et al.*, 2000). These have all been observational. Despite much work on how the emergence of antiviral resistance affects the polymerase protein, only one study by Torresi and colleagues (2002) has sought to characterise how these changes in the overlapping S gene might affect the HBsAg protein conformation. The data presented in this thesis highlight that this issue might be significant.

When lamivudine therapy is withdrawn from a patient with a resistant virus, the virus can revert to express the wild type phenotype (Buti *et al.,* 1997; Chayama *et al.*, 1998; Lau *et al.* 2000; Da Silva *et al.*, 2001). Recent studies in HIV antiviral resistance have, however, shown that for the same phenomena the reversion is often not entire, because the wild type amino acid sequence that the virus returns to may not actually be the original wild type nucleotide sequence, i.e., there may be "scarring" of the third codon by the resistance change (Rob Gifford, UCL, personal communication). When selective pressure is re-applied to the virus, the genetic scarring may pre-dispose the scarred virus to evolve in directions less likely to be the original wild type, unscarred virus. Whether this phenomenon occurs for HBV is an interesting prospect as selective pressure can be applied in both overlapping genes. As the HBV S gene and *pol* overlap, there is a much higher degree of genetic constraint. For a *pol* codon to retain a third nucleotide

mutational "scar", the mutation in the overlapping S gene if non-synonymous would have to be either advantageous or neutral (and any synonymous change in the S gene would be neutral too). Given this, it is expected that this phenomenon will occur at a lower frequency than might be observed for HIV, but it is still a clear possibility. Further work must be undertaken on this subject to determine its frequency and clinical impact in HBV. This issue highlights that even at the nucleotide level, data need to be analysed in respect to both genes.

6.3: Antiviral mutations: implications on the amino acid/protein level

The work encompassed in chapters 3 & 4 of my study was prompted by the work of Torresi *et al.* (2002b) who reported that Lamivudine resistance-associated mutations in isolation and in combination can lead to reduced HBsAg binding to pooled vaccinee anti-HBs. The major findings of my work cannot confirm their proposal that mutations of the conserved YMDD motif in isolation are sufficient to reduce HBsAg antigenicity. Furthermore, my data reveal that specific antiviral-resistance mutations of the YMDD motif, in combination with specific antiviral resistance-associated compensatory mutations can distort specific "a" determinant epitopes even though the mutations are located in different domains. The new data cannot substantiate the specific inference of Torresi *et al.* that reductions in antigenicity observed with mutant rtV173L/sE164D+rtM204V/sI195M HBsAg in particular is due to an altered putative epitope in residues s187-207, though this is due to the limitations of the experiments undertaken. Thus, the idea that these mutations influence distal epitopes requires more consideration. It may be that the both the "a" determinant and the downstream epitopes in s187-207 are affected. The amino acid substitutions studied in this thesis are highlighted in red on a structural model of HBsAg in figure 6.3 below.

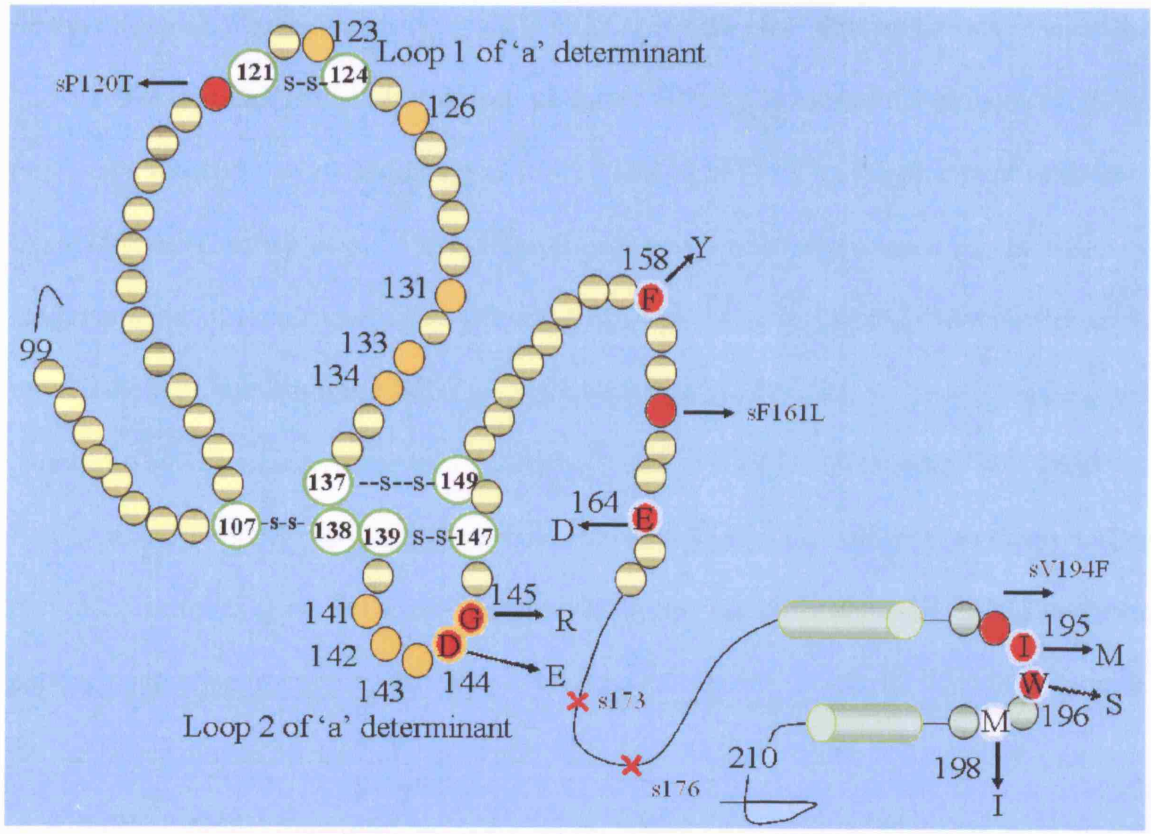


Figure 6.2: A model for the "a" determinant and predicted downstream epitopes. Codons for mutations studied in this thesis are highlighted in red, except those which lie within the putative "buried" trans-membrane region from s167-187 (rtA181V/sL173F, rtA181T/sW172STOP and rtT184S/sL176V) which have been marked with red crosses [Figure after Carman (1997)].

The phenomenon of single amino acid substitutions influencing distant epitopes of HBsAg is not a novel observation, as mutations in the second loop of the "a" determinant are known to influence binding to antibodies recognising the first loop (Waters *et al.*, 1992). The data in the current study did not detect any instances of the first loop abrogation due to second loop mutation. However, there are data that show how distal mutations can affect second loop epitopes, thus supportive of the

observations of Waters and colleagues (1992). It is also clear that mutations outside the "a" determinant can lead to the failure to detect HBsAg in certain diagnostic ELISAs: one study showed that mutations in s110-118 and in s154-158 are associated with such misdiagnosis (Carman *et al.*, 1997), though this is of course dependant on the assay or reagents used. Another study showed that mutations in s118 and s120 are sufficient to reduce the binding of mutant HBsAg to polyclonal goat anti-HBs as well as binding to a panel of 4 MAbs which recognised conformational epitopes between s122-147 (Kfoury-Baz *et al.*, 2001). Moreover, it has also been observed that common vaccine-escape mutations in the S gene, selected through therapeutic HBIG pressure in liver transplant patients, can arise in conjunction with mutations in domains in the MHR that lie outside the "a" determinant, specifically between s106-144, s156-159 and s161-184 (Terrault *et al.*, 1998). Finally, mutations at s116, s118, s120, s159 and s184 have been found associated with vaccine escape in immunised infants from Singapore (Chong-Jin *et al.*, 1999), and mutations at surface residues s105, s110, s112, s118 s119 and s120 have been associated with reduced antibody binding in the following HBsAg assays: MonoLisa AgHBs Plus (Biorad, Marnes Ia Coquette, France), Eti-Mak-3 (Diasorin, Anthony, France) and Vidas HBsAg BioMerieux, Marcy-I'Etoile, France) (Jeantet *et al.*, 2004). It is of note that one study observed that most of the mutations suspected to interfere with immunoassay detection for the Bayer ADVIA Centaur, Ortho VITROS ECi and Roche Elecsys HBsAg assays were present as multiple mutations within and upstream of the "a" determinant (Moreman *et al.*, 2004), suggesting that changes within several regions may contribute to loss of detection by affecting epitope conformation. Nonetheless, it is clear that the principal contributor to HBsAg antigenicity remains the "a" determinant (Howard *et al.*, 1984; Zheng *et al.*, 2004).

The preferred explanation to account for the observations from these previous studies (Waters *et al.*, 1992; Carman *et al.*, 1997; Terrault *et al.*, 1998; Chong-Jin *et al.*, 1999; Kfoury-Baz *et al.*, 2001; Jeantet *et al.* 2004; Louisirirotchanakul *et al.*, 2004; Moreman *et al.*, 2004 Wagner *et al.*, 2004) and the observations in this study is that mutations outwith the "a" determinant can act in synergy to abrogate distant "a" determinant epitopes in the HBsAg protein as has been proposed by Waters and colleagues (1992). They suggest the concept of 'epitope moderation' whereby an amino acid substitution causes a change that is significant enough to trigger a distal epitope change. This concept is at its most credible if substitutions of cysteine residues are involved, as cysteine-cysteine disulphide bonds are particularly important for the maintenance of secondary and tertiary protein structures. Furthermore, if glycosylation sites were lost (such as the N-linked glycosylation site at Asn 146 in the "a" determinant), antigenicity may also be lost, as glycosylation sites can cause HBsAg misfolding (Lu *et al.*, 1997). My studies have detected neither substitutions of cysteine nor asparagine. Drastic loss of antigenicity at distal sites may also be due to substitution of proline, as proline is often located at the turn of a peptide chain in the three-dimensional structure of a protein and therefore critical to protein conformation (Steward *et al.*, 1983; Wallace *et al.*, 1994). Only one proline mutation - the lamivudine resistance-associated compensatory mutation rtT128N/sP120T - was indeed identified in this study, and found to be associated with reduced binding to the MAb H3F5, which recognises the epitope in s131-142, located 11-22 amino acids away from the mutational site. So proline, cysteine and asparagine substitutions cannot account for the frequency with which single and combined mutations affected distal epitopes. Nonetheless, it should be remembered that the typical and well studied immune escape mutation sG145R does not encode proline, cysteine or aspargine substitutions, yet in this study clearly abrogated

both second loop epitopes. This non-conservative change has the potential to substantially alter the conformation of the "a" determinant.

The rtM204V/sI195M mutation when isolated does not alter distal "a" determinant epitopes. Isoleucine, which is substituted for methionine in the S ORF, is aliphatic, and - being neutral, non-polar and hydrophobic - does not form hydrogen bonds. However, isoleucine residues are critically important for ligand binding to proteins, and play central roles in protein stability (Copeland *et al.*, 1984). Methionine is a neutral, non-polar amino acid that is marginally less hydrophobic. It also has a sulphur-containing side chain that is less prone to forming structural interactions; unlike cysteine (which also has sulphur containing side chain) the sulphur side chain of methionine is not highly nucleophilic. Therefore, the mutation from isoleucine to methionine can be considered structurally conservative. This may explain why in isolation it does not alter "a" determinant antigenicity. Nonetheless, this mutation is sufficiently different from wild type to lend itself to interaction with other distal mutated codons upstream in the HBsAg molecule to allow significant conformational change.

One such mutation, the rtV173L/sE164D change, itself did not abrogate any of the MAb epitopes studied. In conjunction with rtM204V/sI195M, however, a complete loss of the D2H5-binding epitope (located between s142-147) resulted. The substitution of glutamic acid for aspartic acid at codon s164 alone may also be considered structurally conservative, as both amino acids are polar and are charged with acidic side groups. Nonetheless, glutamic acid is more acidic than aspartic acid: the pK_a of the γ carboxyl group for glutamic acid in a polypeptide is about 4.3, significantly higher than that of aspartic acid (1.9), which may be due to the inductive effect of the additional methylene group. In this interesting exchange, the two structurally conservative mutations

(rtM204V/sI195M and rtV173L/sE164D) individually exerted no direct effect on the "a" determinant, but when acting in concert induced structural changes of the distal epitope.

Despite the above considerations, structural explanations for these observations remain unsatisfactory. Other workers studying similar phenomena (Waters *et al.*, 1992; Carman *et al.*, 1997; Terrault *et al.*, 1998; Chong-Jin *et al.*, 1999; Kfoury-Baz *et al.*, 2001; Jeantet *et al.* 2004; Louisirirotchanakul *et al.*, 2004; Moreman *et al.*, 2004 Wagner *et al.*, 2004) have also been unable to explain their findings in the context of our current understanding of HBsAg conformation. Models of HBsAg structure, that proposed by Chen *et al.* (1996) based on phage display analysis, and that proposed by Prange & Streek (1995) based on bioinformatics, generally agree to a 'two loop' structure for the "a" determinant. They disagree as to the structure of regions downstream of the "a" determinant, particularly towards the carboxyl terminus of the molecule. This contrast may be due to modelling efforts being primarily applied to resolve the "a" determinant structure. The findings of Paulij *et al.* (1999), following the discovery of a novel monoclonal antibody that is able to bind to residues s178-186, further supports the view that the 2 models are inadequate in predicting the structure of HBsAg downstream of the "a" determinant, as both models predict the region between s178-186 to be buried within the lipid bilayer.

The data of this thesis supports the approach of considering HBsAg antigenicity in a way that is less focused on the "a" determinant and which considers regions out with it as relevant. This has already been proposed by Carman and Wallace (1997) who proposed the concept of the major hydrophilic region (MHR), which covers s99-169. Given the data presented in this study, there is also strong evidence that regions located downstream from the MHR and the "a" determinant are also critical for HBsAg

antigenicity. More work is needed to define how mutational change in s187-207 affects HBsAg conformation and the presentation of "a" determinant epitopes.

6.4: Virological and epidemiological considerations

All the mutants studied in this thesis are replication-competent, and all have been observed in patients (Ling *et al.*, 1996; Ling and Harrison 1996; Tipples *et al.*, 1996; Ogata *et al.*, 1999; Lok *et al.*, 2000; Torresi *et al.*, 2002b; Delaney *et al.*, 2003; Tenney *et al.*, 2004 and Hadziyannis *et al.*, 2005). It would be experimentally difficult to separate selective advantage obtained upon mutation in both S and *pol* genes. If a virus bearing the lamivudine resistance mutation rtM204V/sI195M were also to accumulate the compensatory mutation rtV173L/sE164D, would that virus now bear HBsAg that evades the patient's anti-HBs? And, would that evasion have an impact upon development of infection? Conversely, if a virus bearing the vaccine-escape mutation sG145R were to acquire the lamivudine resistance-associated mutation rtM204V/sI195M, would its HBsAg, as was observed in this study, become more susceptible to neutralization by the patient's anti-HBs? Seeking answers to these questions is outside the scope of this study. It would require experiments designed to separate the effects of single nucleotide substitution mutations upon the overlapping gene.

The potential of HBV bearing antiviral resistance mutations to evade anti-HBs and replicate within immunised individuals must be considered. This may be an alternative vehicle for the selection and transmission of immune escape variants. Typically, drug-resistant mutants are less replication-competent than wild type virus (Melegari *et al.*, 1998), but the development of resistance compensatory mutations can restore replicative

capacity to near wild type levels. Work in this thesis highlights that such resistance compensatory mutations can act to abrogate "a" determinant epitopes. The removal of lamivudine therapy results in a return to wild type status (Buti *et al.,* 1997; Chayama *et al.*, 1998; Lau *et al.* 2000; Da Silva *et al.*, 2001). Hence the transmission of drug-resistant mutants has been rarely documented (Thibault *et al.*, 2002) and the results of chapter 5 of this thesis showed that they were not detected in incident UK infections in the period of 1997-2001, though this was a time in which antiviral drugs were not in widespread use. But there are some caveats to this consideration, as minor populations were not studied in this thesis and now there are some doubts if wild type reversion upon the withdrawal of therapy is a return to true wild type (Rob Gifford, UCL, personal communication). It must be asked whether drug resistant mutant transmissions are being masked due to a reversion to a "scarred wild type" sequence.

Secondly, it has also been shown that HIV which has become resistant to protease inhibitors does not always return to wild type when therapy is withdrawn. The virus can maintain resistant compensatory mutations for up to 4 years despite the loss of primary resistant mutations (van Marseeven *et al.* 2007). Such virus was unable to return to wild type due to the genetic burden of the accumulated compensatory mutations. Although it is clear that lamivudine-resistant HBV does return to wild type upon withdrawal of therapy (Buti *et al.,* 1997; Chayama *et al.*, 1998; Lau *et al.* 2000; Da Silva *et al.*, 2001), this full return has not been shown for adefovir or entecavir. Moreover, evidence derived from very sensitive mutational assays has detected lamuvudine resistant variants before therapy has commenced in treatment naïve patients (Kobayashi *et al.*, 2001; Kirishima *et al.*, 2002; Shin *et al.*, 20031; Heo *et al.*, 2004; Leon *et al.*, 2004), thus these assays similarly should be applied to those instances where lamivudine has been withdrawn The work of van Marseeven and colleagues (2007) highlights that this

phenomenon must be studied for each individual mutation as there is the theoretical possibility that in some instances antiviral resistant HBV might be unable to return to wild type despite the lack of selective pressure. By contrast, the transmission of immune escape variants that are transmissible and are able to cause chronic liver disease has been well documented (Thakur *et al.*, 2005). Yet it is shown that immune escape variants such as sG145R can function as lamivudine resistance-associated compensatory mutations (Torresi *et al.* 2002a). Antiviral resistance and immune escape are more closely tied issues than previously thought and these issues must be taken into account when considering the transmission and epidemiology of such mutants.

In chapter 5 of this thesis, it is argued that the conditions present in the UK are not amenable to the regular transmission of antiviral resistant variants. Low HBV incidence, and well-regulated antiviral therapy with the availability of alternative drugs make selection of such mutants less likely. As discussed above, there are grounds to suspect that the transmission of drug resistance mutations may be masked in minor populations through various means, but this has not been confirmed for HBV so far. The transmission of immune escape variants which may then act as antiviral resistance compensatory mutations is also less likely due to the lack of universal immunisation program and again the low incidence of HBV.

If these very same issues for the developing world and in particular Africa are considered, however, the picture painted is somewhat more worrying. There is a high childhood incidence of HBV in Africa that leads of a high rate of chronic carriage in adulthood (Hino *et al.*, 2001). There are approximately 50 million chronic carriers of HBV in Africa and in sub-Saharan African the chronic carriage rate ranges from 9-20% (Kiire, 1996). Owing to lack of resources it would be expected that few receive antiviral

therapy specifically for HBV chronicity. Some countries have implemented universal vaccination programmes (Lavanchy, 2004). The situation for HBV in combination with the high rate of HIV incidence is a concern. HIV infected patients will experience immune suppression and many are treated with multi-drug therapy including the zidovudine-3TC (lamivudine) combination. For example in Botswana 39% of the 730,000 adults between the ages of 15-49 are HIV infected with around 42,000 of them currently receiving antiviral therapy (Vardavas and Blower, 2007). A recent meta-analysis based on patient self reports suggests that adherence in Africa may be better than North America (Mills *et al.*, 2006). However, there are often concerns regarding poor antiviral therapy adherence in resource poor areas due to intermittent supply (Weiser *et al.*, 2003). Moreover it is clear that for HIV, the transmission of drug resistant virus in developed countries is quite common (Wensing and Boucher, 2003).

Thus, in Africa, there is a population with high HBV incidence, HBV replication tolerance through childhood exposure, partial immunity and the selective pressure of natural and vaccine-induced anti-HBs, HIV-induced immune suppression allowing increased viral replication, and the potential for poor adherence to lamivudine containing antiviral regimens to be disrupted due to poor supply. These conditions make the selection and transmission of antiviral resistant variants which can evade anti-HBs much more likely than for those conditions present in the UK. Future surveillance and clinical studies in the regions would do well to bear in mind these considerations. It is of concern for those countries which have implemented vaccination campaigns that there is an alternative vehicle for the selection of immune escape mutants other than the already well understood routes. Epidemiological predictions of the emergence of immune evasion variants of HBV in these regions might need to be considered to accommodate these phenomena.

6.5: Subversion of diagnostics and antiviral therapy

6.5.1: Subversion of diagnostics

The detection of the presence of HBsAg in patient sera is the primary test for the presence of HBV. Different commercially available HBsAg detection assays have different capabilities in detecting HBsAg mutants (Carman *et al.*, 1997; Ireland *et al.*, 2000; Jeantet *et al.*, 2004; Levicnik-Stezinar *et al.*, 2004; Louisirirotchanakul *et al.*, 2004; Moreman *et al.*, 2004; Wagner *et al.*, 2004). Increasingly, however, ELISAs are based less on HBsAg capture by single MAbs that recognise the "a" determinant, and more on multiple monoclonal and polyclonal components, or on MAbs that bind to known HBsAg mutants (Jolivet-Reynaud *et al.*, 2001). However, it has been suggested that such detection may be at reduced assay sensitivity (Seddigh-Tonekaboni *et al.*, 2000). Thus, the problem is a diminishing one. PCR analysis and analysis of other viral markers can also aid diagnosis. Although the use of antiviral therapy to treat chronic HBV is ongoing, the improvements made to the current generations of ELISAs means that misdiagnosis is unlikely.

6.5.2: Subversion of antiviral therapy

There are increasingly more antiviral therapies available for the treatment of chronic HBV with more in clinical trial development (see table 1.1). Throughout this thesis, specific mutations have been referred to as "lamivudine-resistant" or "entecavir resistance-compensatory", *etc.* But this classification is becoming increasingly difficult, as it is apparent that there is a considerable degree of cross-resistance between current

antivirals. The two most recent drugs for HBV chronic infection, tenofovir (in phase III clinical trials 2005) and entecavir (licensed in 2006), are associated with the development of antiviral resistance mutations appearing in the context of pre-existing classic lamivudine resistance mutations (Tenney *et al.*, 2004; Sheldon *et al.*, 2005). Table 6.1 below indicates cross reactivity for current antivirals and their resistance mutations.

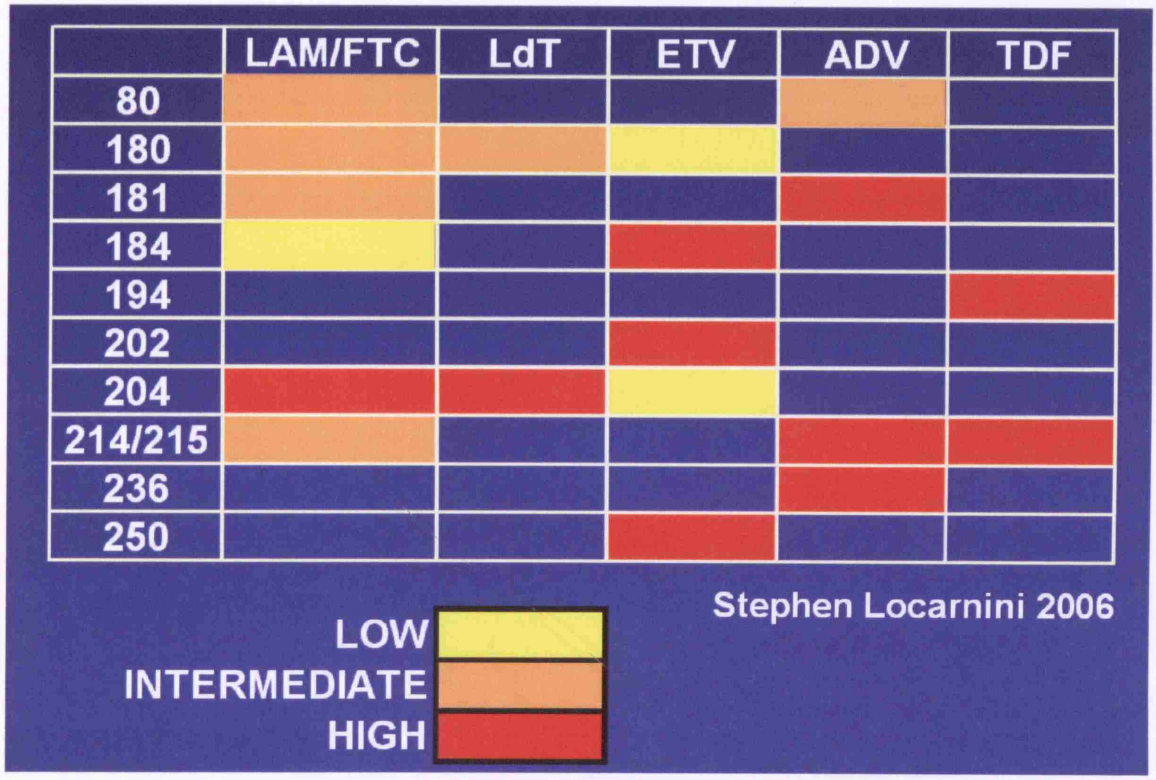


Table 6.1: Cross resistance of mutations at reverse transcriptase codons (numbered) to various antivirals for HBV. LAM = Lamivudine, LdT = Telbivudine, ETV = Entecavir, ADV = Adefovir, TDF = Tenofovir. HIGH = Well defined mutation with high IC_{50}. INTERMED (Intermediate) = Well defined resistance compensatory mutation leading to high IC_{50} in conjunction with other mutations OR mutation leading

to moderate increase in IC_{50}. LOW = Minor and less well studied resistance compensatory mutation. (Figure reproduced with permission, Stephen Locarnini 2006).

Despite concerns over the use of sequential monotherapy in its potential to generate multidrug resistance (Mutimer *et al.*, 2000b), this approach to therapy has been advocated by the National Institute of Clinical Excellence (NICE) in the UK. This is of concern, though it should be borne in mind that as yet many combinations of antivirals such as lamivudine + adefovir have failed to show synergistic effect (Angus *et al.*, 2003, Villneuve *et al.*, 2003). Given the number of antivirals in development and the relatively high number of currently licensed antivirals, there is hope that synergistic combinations will be found. However, as all these drugs are nucleoside analogues which function in the same manner, it is arguable that synergistic combinations will never be found. Thus, sequential monotherapy might be administered frequently in the future, raising the potential for development of multiple mutated viruses to develop. As has been described in this thesis, virus bearing multiple *pol* mutations can strongly perturb the "a" determinant. It is therefore considered that the possibility of immune evasive virus developing as a result of antiviral therapy is an increasing one.

6.6: Is this phenomenon HBV specific?

According to one of the dogmas of the genetic code, each gene is responsible for the coding of one protein/polypeptide. This hypothesis was proposed by Beadle and Tatum in the 1940s and is called the "one gene, one protein" hypothesis. Barrell and colleagues (1976) first gave evidence that suggests the possibility of overlapping of genes in the bacterial virus φ X I74. Overlapping genes have now been found in other viruses, but is most widespread in small viruses such HBV, this might be because of the limitations of the quantity of nucleic acid that can be enclosed in the small viral capsids. However, overlapping (sometimes referred to as nested) genes can be found in herpes viruses (e.g.

HSV-1, CMV), papillomaviruses (e.g. HPV-16, SV40), senadiviruses and in the accessory genes of retroviruses (e.g. HIV-1). In these examples, a huge variety of genes coding for different viral proteins are overlapped. In any instances where envelope/surface or core/capsid genes overlap there is the potential for mutational change to affect B-cell or T-cell epitopes. Other than for HBV there are no clear examples of iatrogenically induced mutations which affect overlapping ORFs to produce a virus of a phenotype that may be clinically detrimental. The most likely scenario may be via the accessory genes of HIV-1. They code for unique viral replicatory functions which are still untapped as a target for antiviral therapy (Mezei & Minarovits, 2006). In this light, the accessory proteins *Rev* and *Tat* of HIV are of particular concern as both are entirely overlapped by the *env* gene which codes for the viral surface glycoprotein.

6.7: Future work and study modifications

There are a number of areas of investigation that could stem from what has been achieved in this study. A number of which have been alluded to in the above discussion, but which will be touched upon again. Firstly (6.7.1), there are questions that can be answered using the same approach as outlined in this study. Secondly (6.7.2), there are the completely new studies which could arise from the study data.

6.7.1: New studies which could be undertaken using the same methods

Novel resistance mutations must be studied

The easiest extension upon this study would be to continue to study more antiviral resistance mutations which overlap into the *S* gene. New antiviral resistance-associated

mutations in the *pol* gene of the HBV genome against nucleoside analogues are continually reported (Bartholomeusz, & Locarnini, 2006) and at the time of the writing up of this thesis, new mutations associated with resistance to tenofovir (Sheldon *et al.*, 2005), and adefovir (Hadziyannis *et al.,* 2005) had appeared in the literature. It is necessary that as new antiviral resistance mutations arise, that their potential for antibody evasion is characterised.

African genotype virus must be studied

Secondly, studies using a different genetic backbone would be of value. This study used genotype A, predicting a serotype *adw2* virus, but given the strong concerns outlined for the selection of immune evasive drug resistant virus in Africa in HIV prevalent areas, studies using a common African genotype virus (such as genotype Aa and genotype E) are now necessary.

Other mutations of the polymerase YMDD motif must be studied

The main finding of this study was that an interaction between mutations within the conserved YMDD motif and mutations further upstream (up to 50 amino acids away) can abrogate "a" determinant epitopes. While my study focussed on the rtM204V/sI195M mutation, it remains to be determined if other mutations in the YMDD motif, e.g., rtM204I/sW196S and rtM204I/sW196L, also have the potential to disrupt "a" determinant conformation.

Current models of HBsAg structure need to be considered

The results of this study also cause us to question the precision of current models of HBsAg conformation in the regions downstream of the "a" determinant. The observations of this thesis make it necessary to review current models. It has been

revealed that some distal epitopes involved in antiviral resistance may be critical for conformation, yet a similar study could be undertaken by disregarding antiviral resistance and by simply performing mutagenesis within specific regions of HBsAg and noting their performance in immunoassays.

6.7.2: Studies which could be undertaken using other methods

Necessity for a MAb directed to s187-207

The greatest improvement for this work would have been the availability of MAbs directed towards the putative surface epitope at s187-207. By looking at how MAb binding to this region related to the results obtained it might have been possible to more clearly define the involvement of this region on "a" determinant conformation and confirm the hypothesis of Torresi and colleagues (2002) that mutations of the YMDD motif affect this downstream surface epitope. Unfortunately no such MAb has yet been developed. The production of such a MAb would greatly benefit our understanding of the role of the s187-207 epitope in "a" determinant conformation.

Does resistant virus always revert to wild type upon withdrawal of therapy?

Another study which may arise from this work is the potential for HBV bearing antiviral resistance mutations to revert to an amino acid wild type, but nucleotide non-wild type state as described by Gifford and colleges for HIV (Rob Gifford, UCL, personal communication). As discussed above, this phenomenon may also be important for HBV, especially for the potential for masked transmission of HBV drug resistance. This could easily be undertaken with sequence data available in publicly available HBV databases, such as that described by Gnaneshan et al. (2007). The phenomenon of

compensatory mutation fixation as described by van Marseveen and colleagues (2007) for HIV must also be addressed for HBV.

What is the clinical impact of resistant immune evasive virus developing?

It was beyond the remit of this study to determine how the selection of mutations in both S and polymerase ORFs *in vivo* might affect clinical outcomes in an infected patient. There have been a number of clinical studies which have observed the emergence of mutations such as these (Tipples *et al.*, 1996; Bartholomew *et al.*, 1997; Terrault *et al.*, 1998; Ogura *et al.*, 1999; Shields *et al.*, 1999; Lok *et al.*, 2000); however, these are the exceptions to the rule. It is still common for researchers to report antiviral resistance mutations in *pol* only, despite recommendations to the contrary (Struveyer *et al.* 2001). Studying both genes will allow phenotypic work for both reverse transcriptase and HBsAg to be more easily undertaken and we might quantify the potential of such mutations to both subvert therapy and undertake immunological escape.

If we review the suggested proposed future work outlined by looking at the titles of section 6.7 we get a flavour of the impact of the work produced in this thesis: *Novel resistance mutations must be studied, African genotype virus must be studied, Other mutations of the polymerase YMDD motif must be studied, Current models of HBsAg structure need to be considered, Necessity for a MAb directed to surface residues 187-207, Does resistant virus always revert to wild type upon withdrawal of therapy? What is the clinical impact of resistant immune evasive virus developing?* A more ordered summary of the above might be: a phenomenon of drug resistance leading to immune evasion has been outlined and precisely defined. However, we cannot predict how the many other resistance mutations will behave especially at the YMDD motif of *pol*.

There is grave concern that this phenomenon may occur most frequently in Africa but we must first define it in studies using African virus. Moreover, the data cause us to doubt the currently understood conformation of HBsAg epitopes. The unusual genetic architecture of HBV may constrain the evolution of HBV but there are many parallels and lessons to be learned from HIV; thus, it is questioned if HBV always reverts to true wild type upon withdrawal of therapy. The importance of all such studies can only be realised when we uncover the true clinical impact of the development of mutations which affect both S and *pol* genes.

The data in this thesis highlight that even once vaccine and drug has developed the story does not end. It is common public perception that once vaccine has been raised or a drug has been developed that a disease becomes "cured". Here, it is evident that iatrogenic interventions may produce unconsidered side affects and serve as a reminder that they must be continually re-assessed and incorporated into existing paradigms in order that both treatment and paradigm may be improved. Yet, these improvements are academic if they are not effectively administered to those in need.

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APPENDIX A1

COMPLETE RECORD OF OUTCOMES OF HBsAg BINDING ANALYSES USING P2D3, H3F5, D2H5, GE34/36 AND HORSE POLYCLONAL ANTI-HBS FROM CHO CELLS TRANFECTED WITH HBV S GENE MUTANT pBK-CMV[smallS] CONSTRUCTS

Figures A1.1 to A1.144 commence overleaf.

Figure A1.1 – Mean binding ratios of rtM204V/sI195M mutant HBsAg in single monoclonal, multiple monoclonal and polyclonal capture ELISAs.

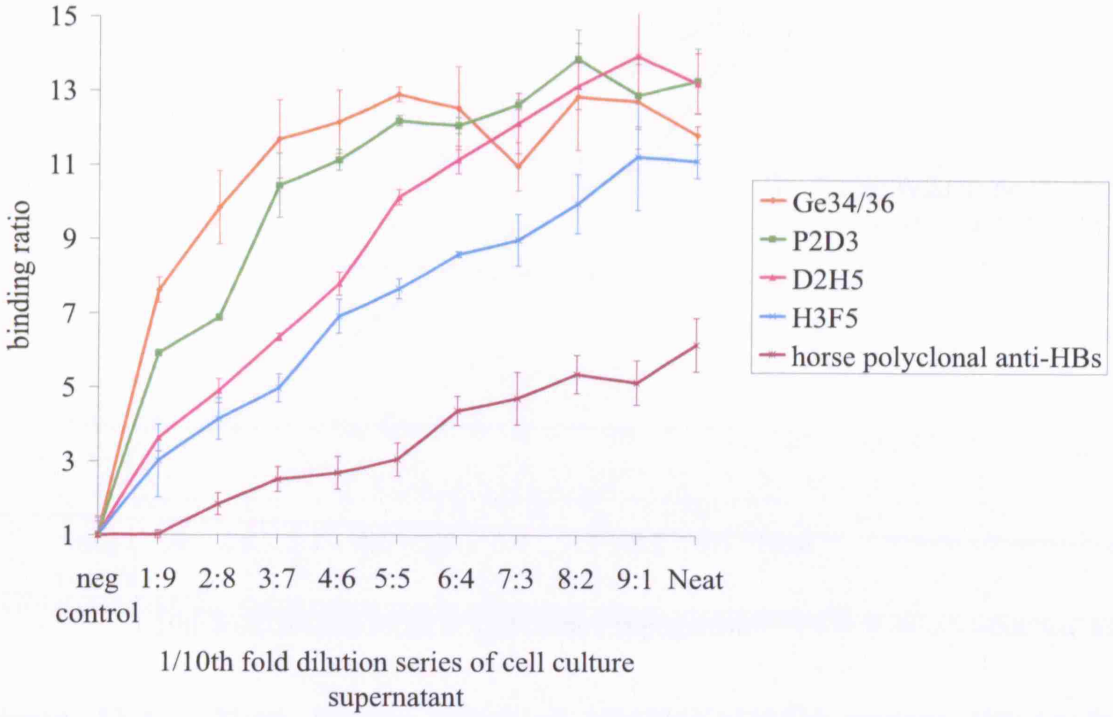


Figure A1.2 – Mean binding ratios of rtM204V/sI195M mutant HBsAg in monoclonal P2D3 capture ELISA.

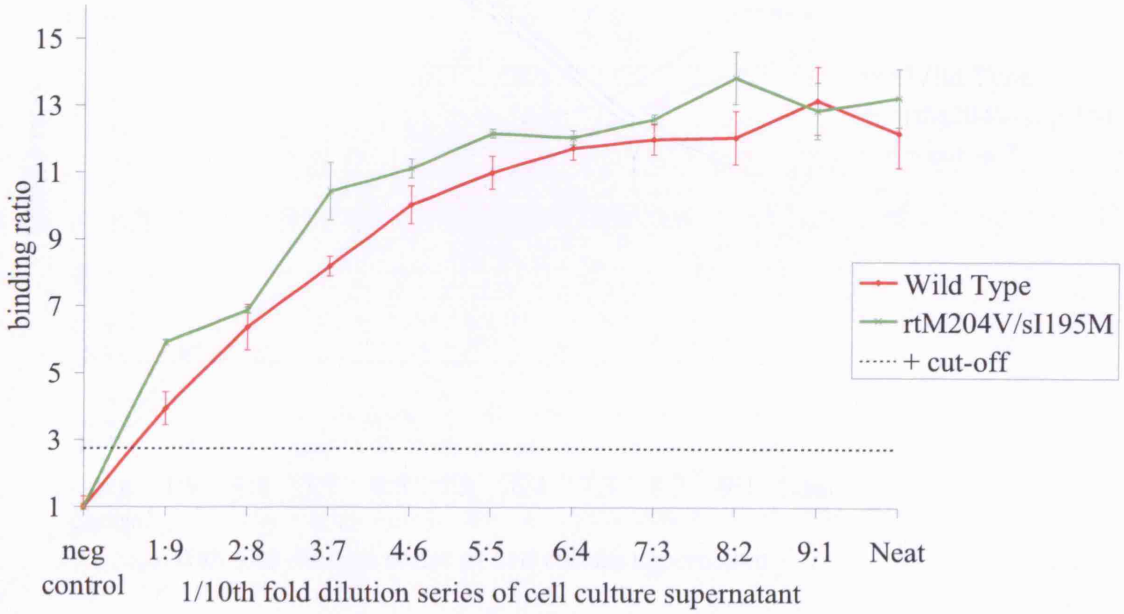


Figure A1.3 – Mean binding ratios of rtM204V/sI195M mutant HBsAg in monoclonal D2H5 capture ELISA.

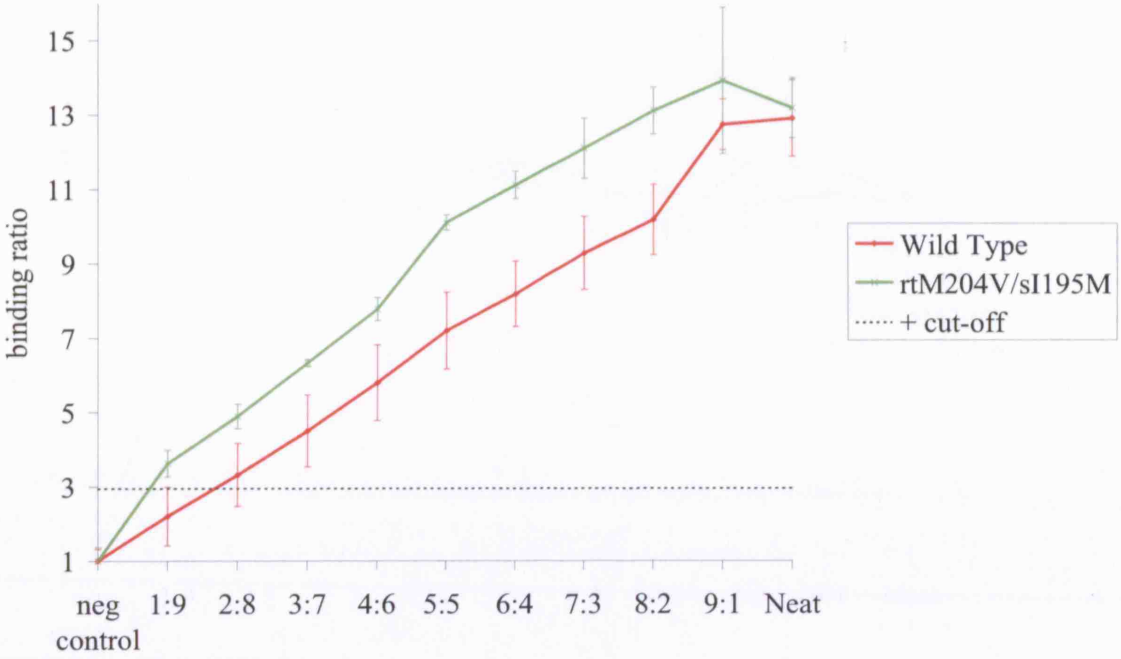


Figure A1.4 – Mean binding ratios of rtM204V/sI195M mutant HBsAg in monoclonal H3F5 capture ELISAs.

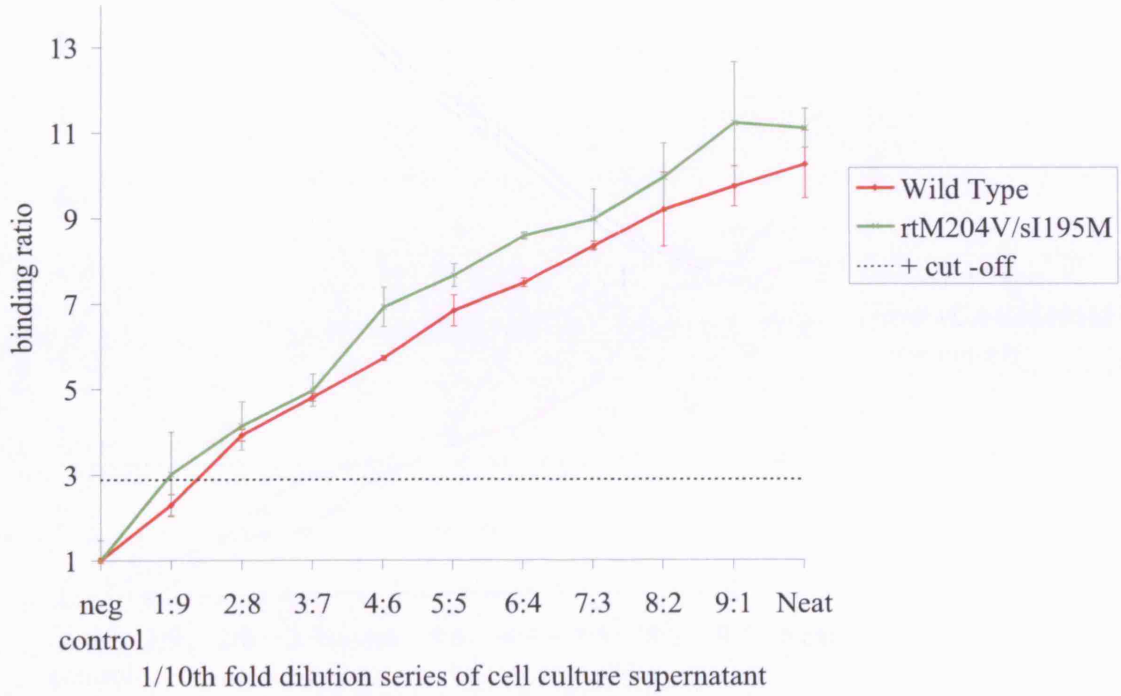


Figure A1.5 – Mean binding ratios of rtM204V/sI195M mutant HBsAg in Ge34/36 format capture ELISA.

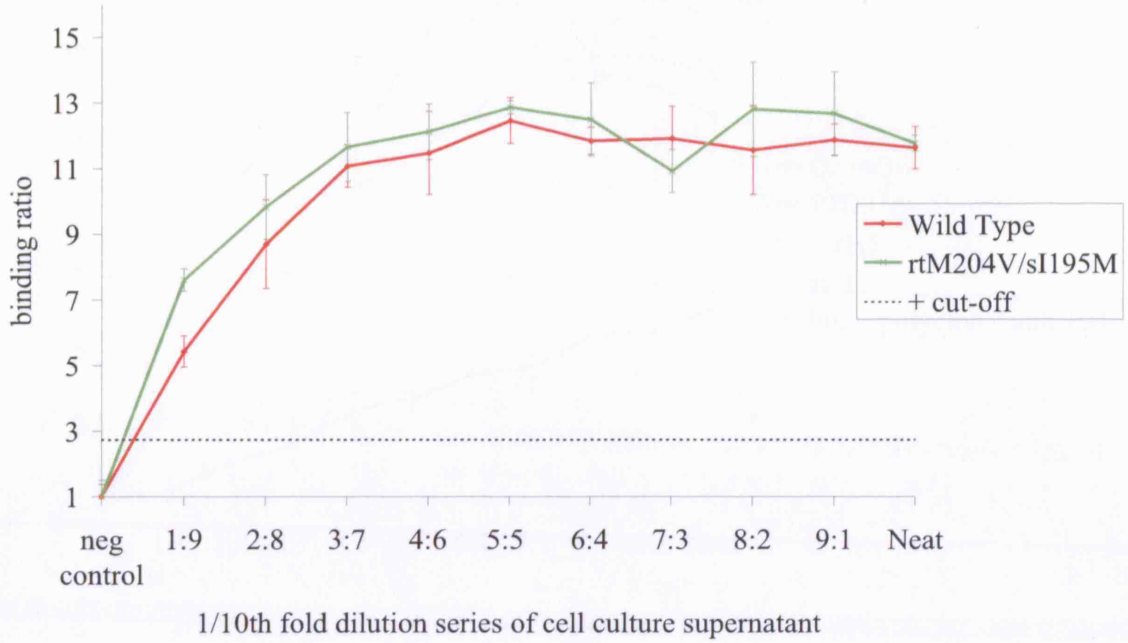


Figure A1.6 – Mean binding ratios of rtM204V/sI195M mutant HBsAg in horse polyclonal anti-HBs capture ELISA.

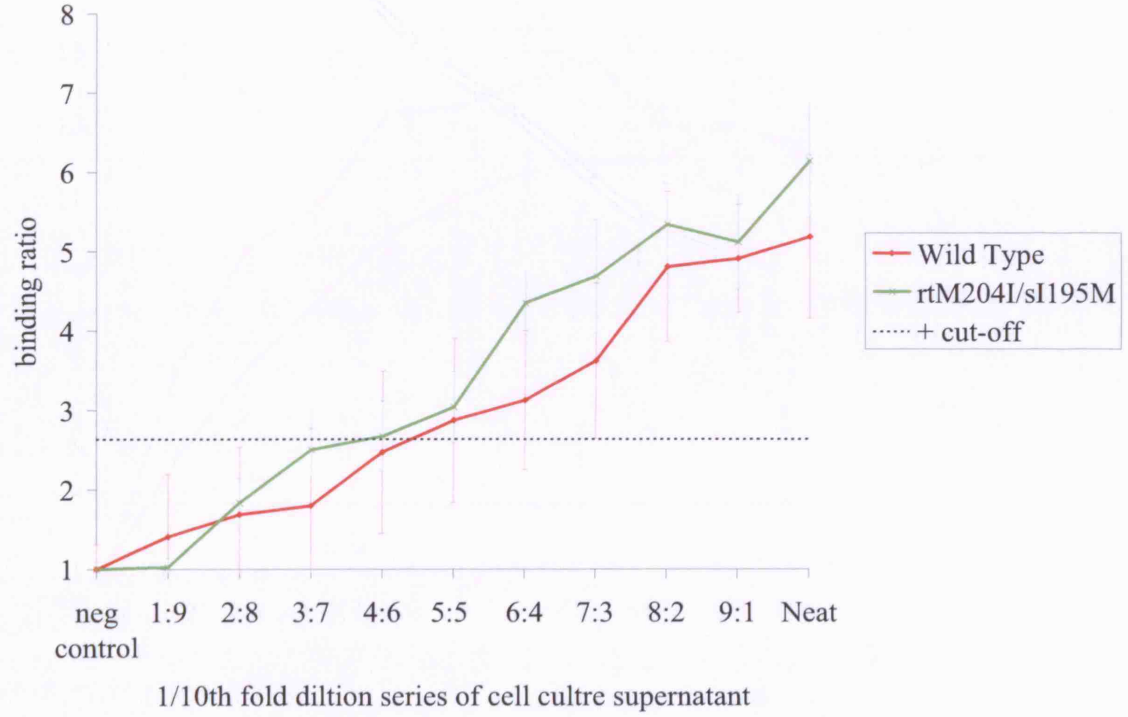


Figure A1.7 – Mean binding ratios of rtM204I/sW196S mutant HBsAg in single monoclonal, multiple monoclonal and polyclonal capture ELISAs.

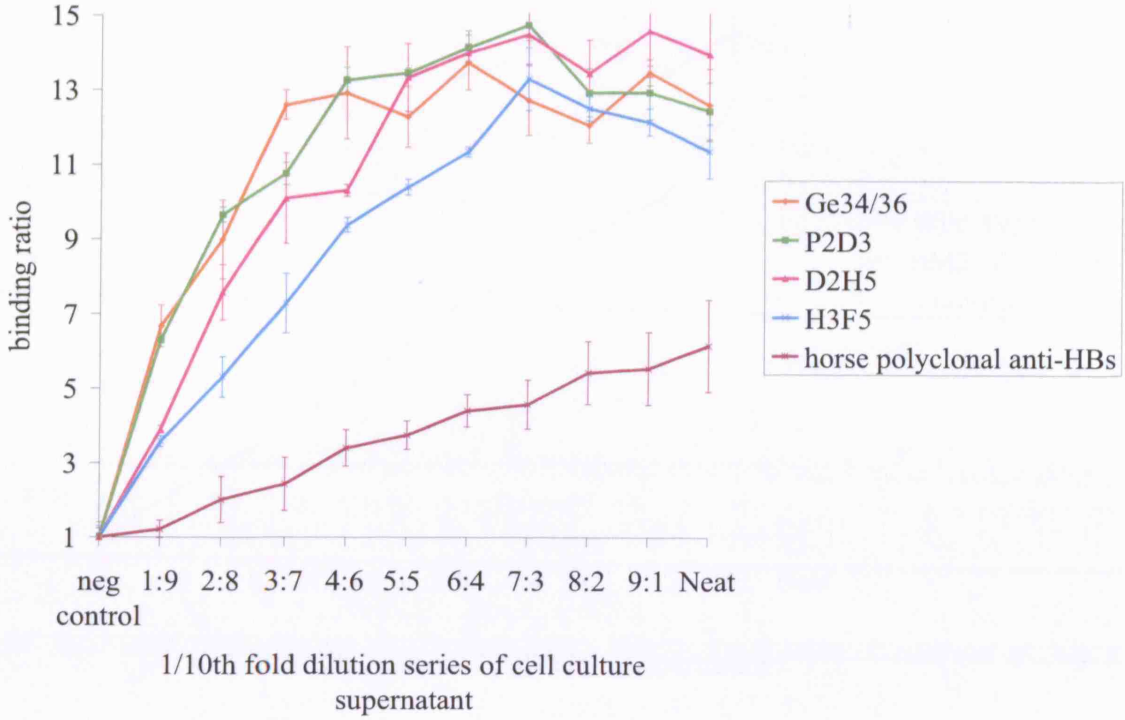


Figure A1.8 – Mean binding ratios of rtM204I/sW196S mutant HBsAg in monoclonal P2D3 capture ELISA.

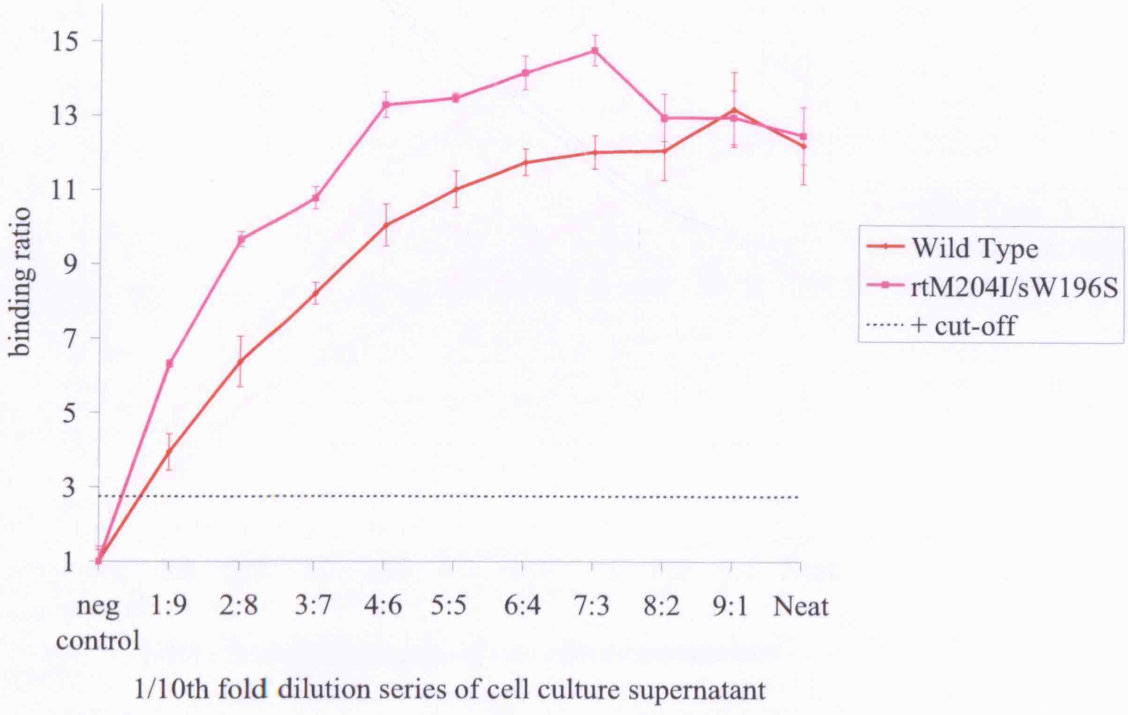


Figure A1.9 – Mean binding ratios of rtM204I/sW196S mutant HBsAg in monoclonal D2H5 capture ELISA.

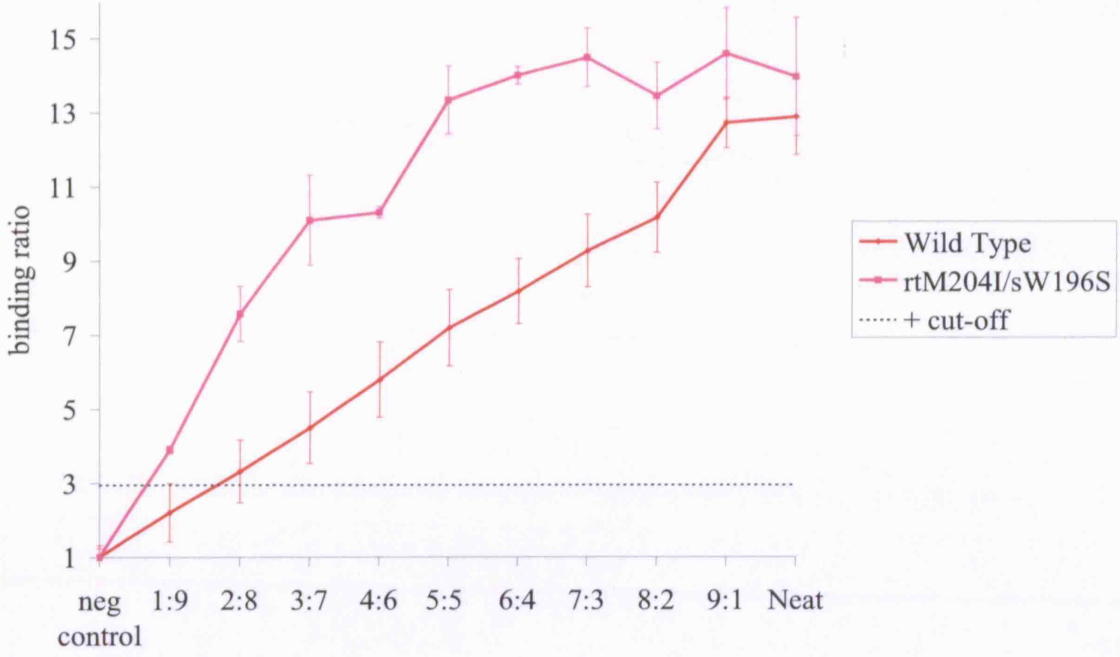


Figure A1.10 – Mean binding ratios of rtM204I/sW196S mutant HBsAg in monoclonal H3F5 capture ELISA.

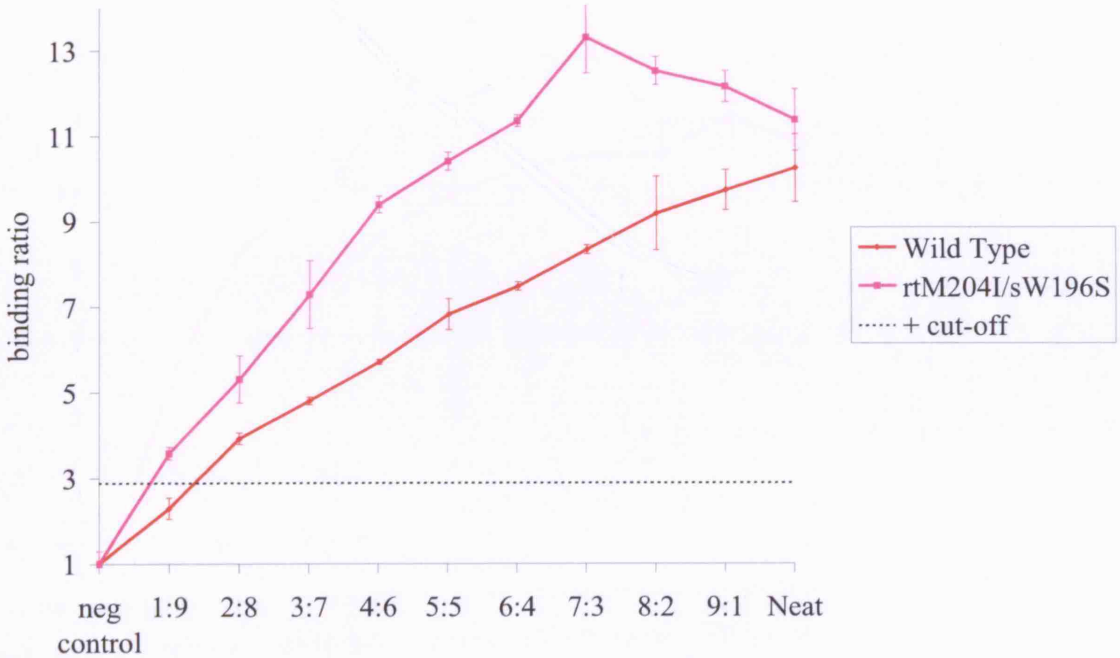


Figure A1.11 – Mean binding ratios of rtM204I/sW196S mutant HBsAg in Ge34/36 format capture ELISA.

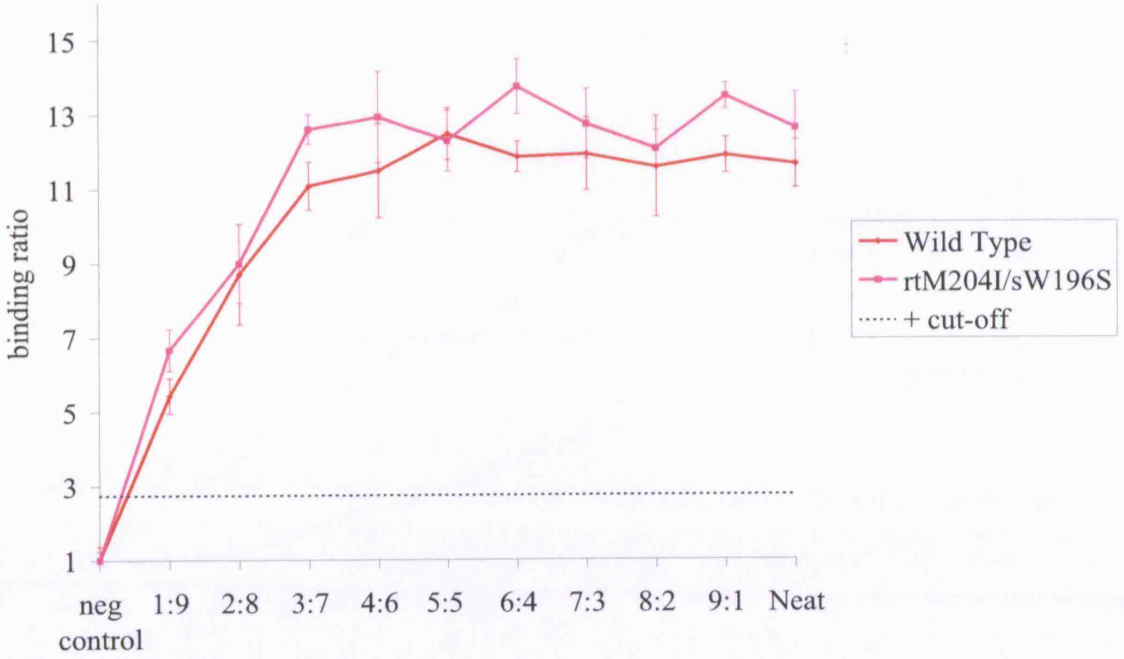


Figure A1.12 – Mean binding ratios of rtM204I/sW196S mutant HBsAg in horse polyclonal anti-HBs capture ELISA.

Figure A1.13 – Mean binding ratios of rtM204I/sW196L mutant HBsAg in single monoclonal, multiple monoclonal and polyclonal capture ELISAs.

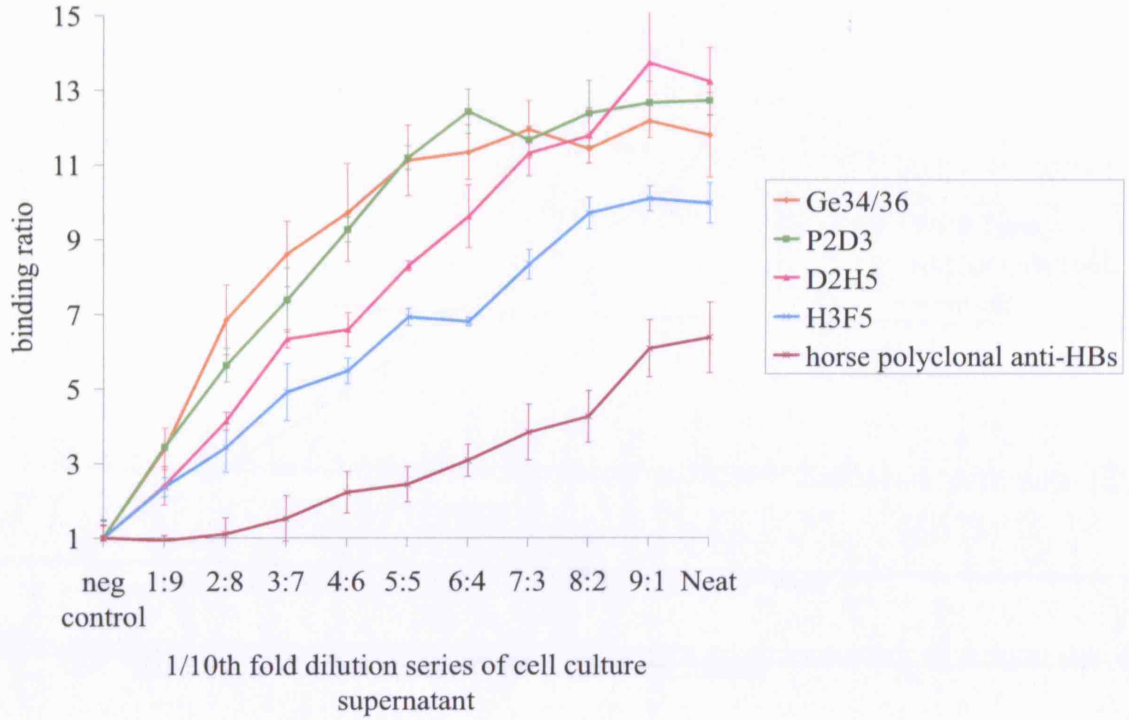


Figure A1.14 – Mean binding ratios of rtM204I/sW196L mutant HBsAg in monoclonal P2D3 capture ELISA.

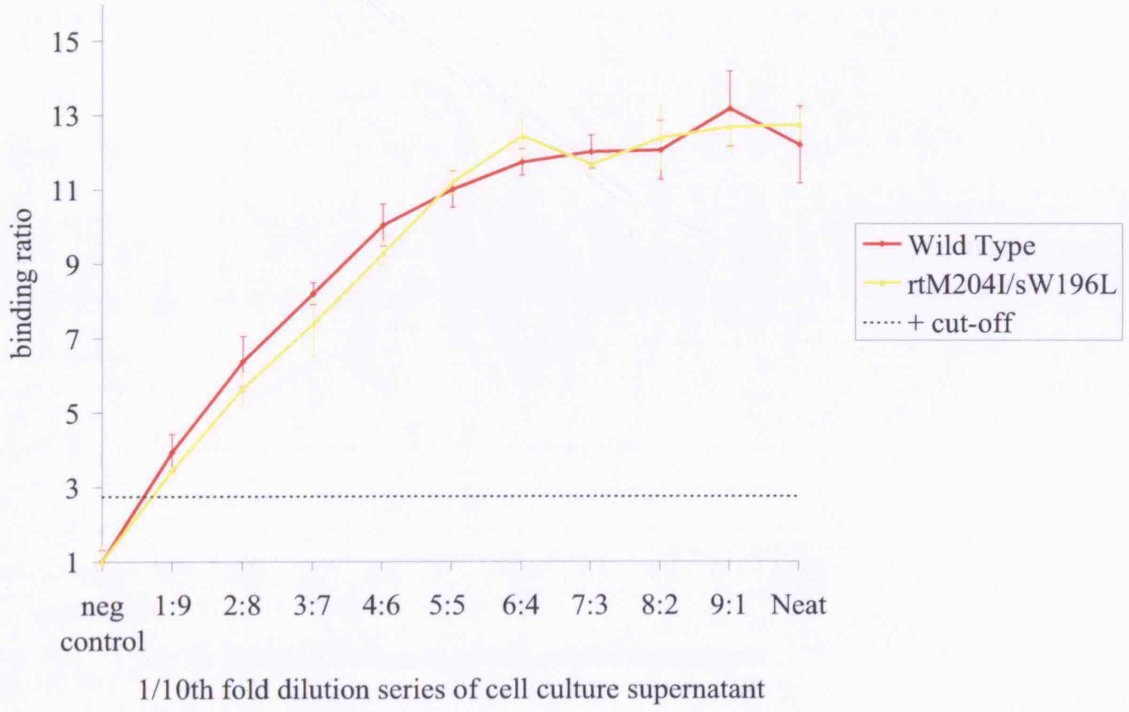


Figure A1.15 – Mean binding ratios of rtM204I/sW196L mutant HBsAg in monoclonal D2H5 capture ELISA.

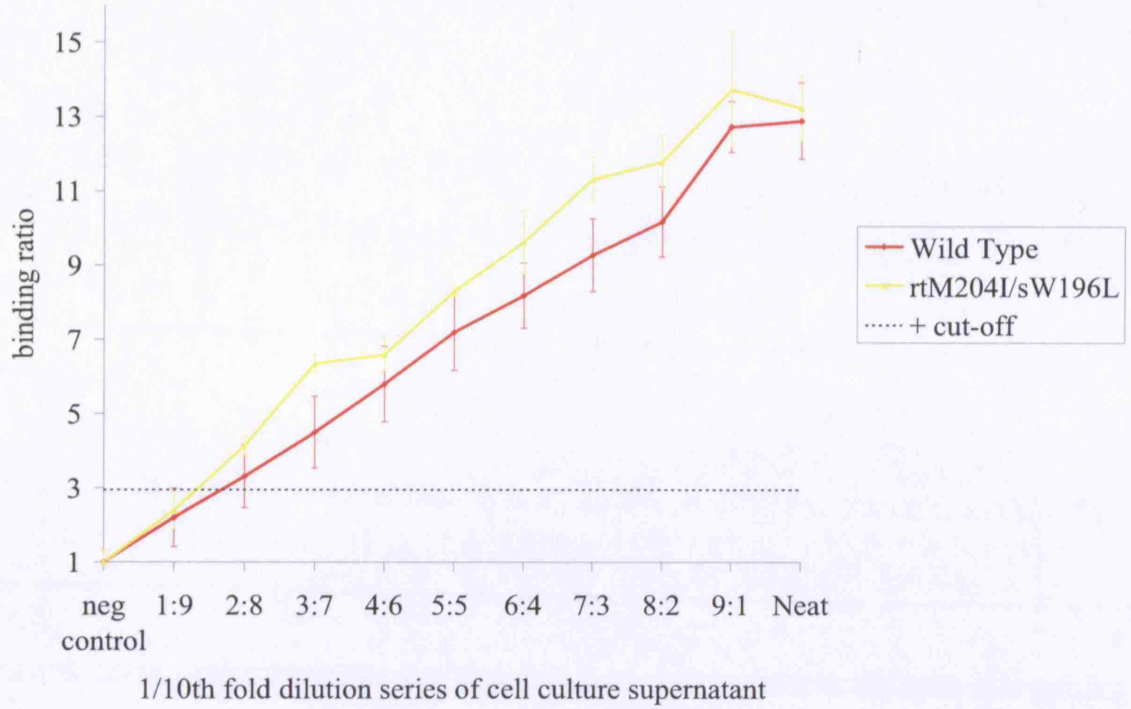


Figure A1.16 – Mean binding ratios of rtM204I/sW196L mutant HBsAg in monoclonal H3F5 capture ELISA.

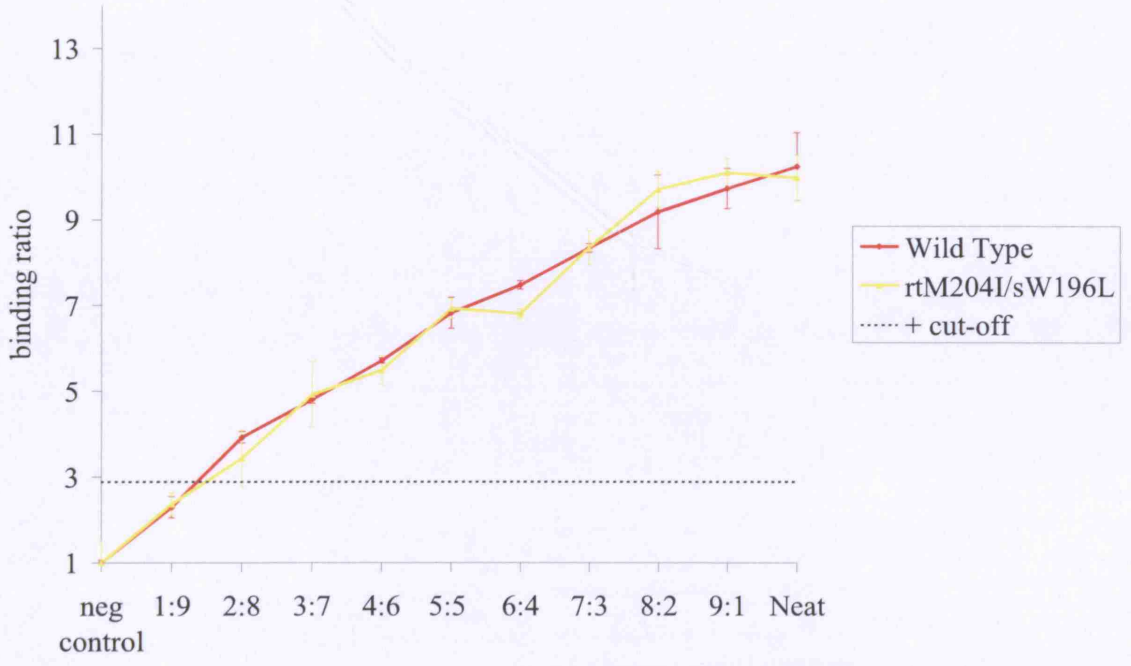


Figure A1.17 – Mean binding ratios of rtM204I/sW196L mutant HBsAg in Ge34/36 format capture ELISA.

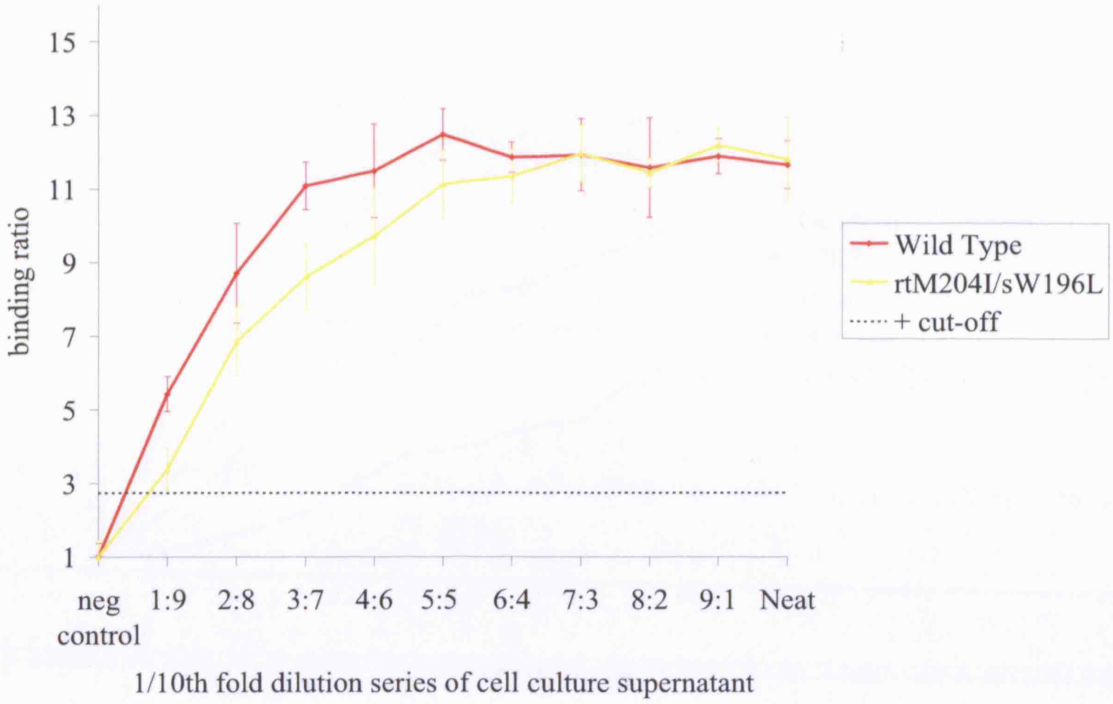


Figure A1.18 – Mean binding ratios of rtM204I/sW196L mutant HBsAg in horse polyclonal anti-HBs capture ELISA.

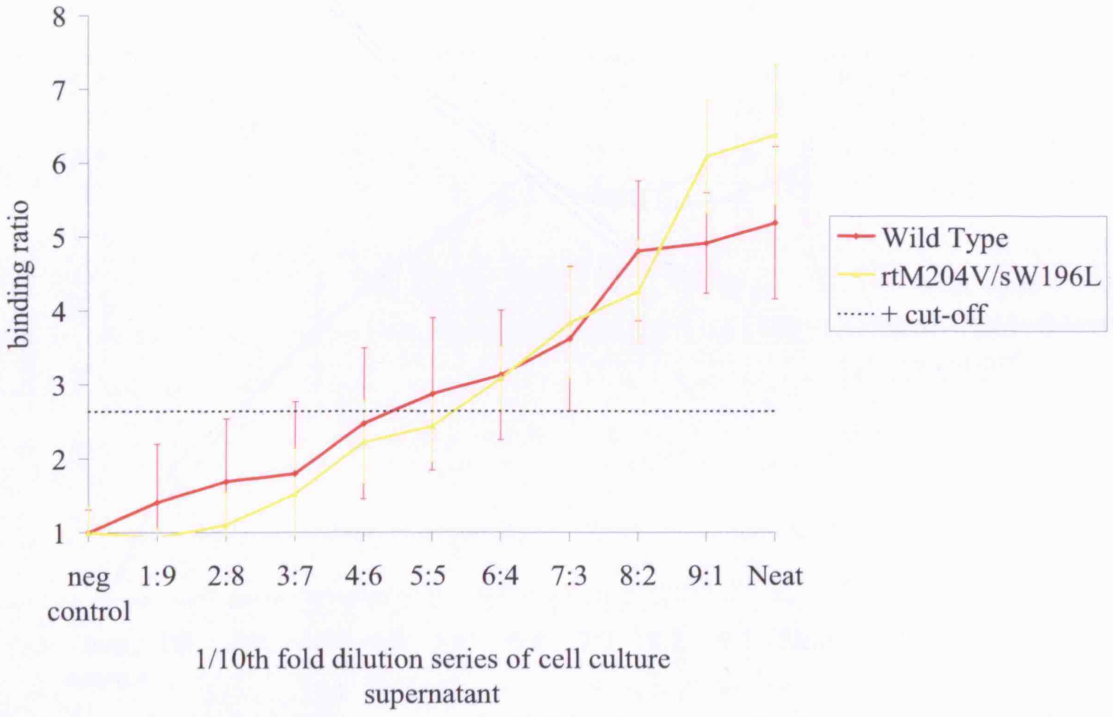


Figure A1.19 – Mean binding ratios of rtL180M/sSilent mutant HBsAg in single monoclonal, multiple monoclonal and polyclonal capture ELISAs.

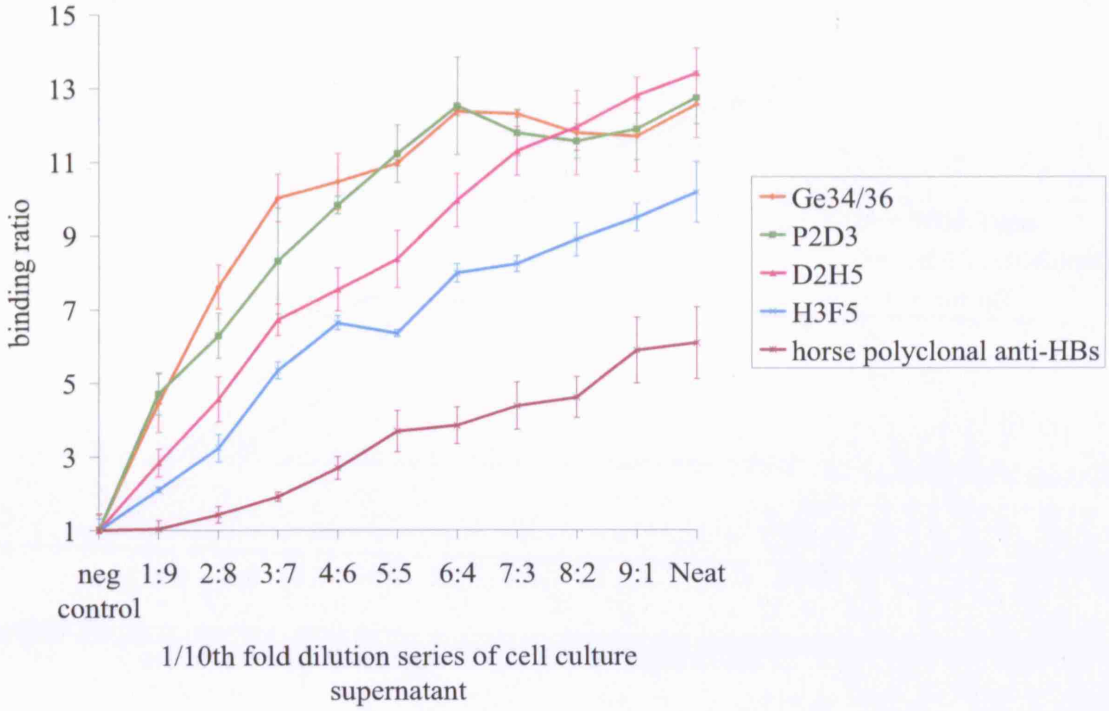


Figure A1.20 – Mean binding ratios of rtL180M/sSilent mutant HBsAg in monoclonal P2D3 capture ELISA.

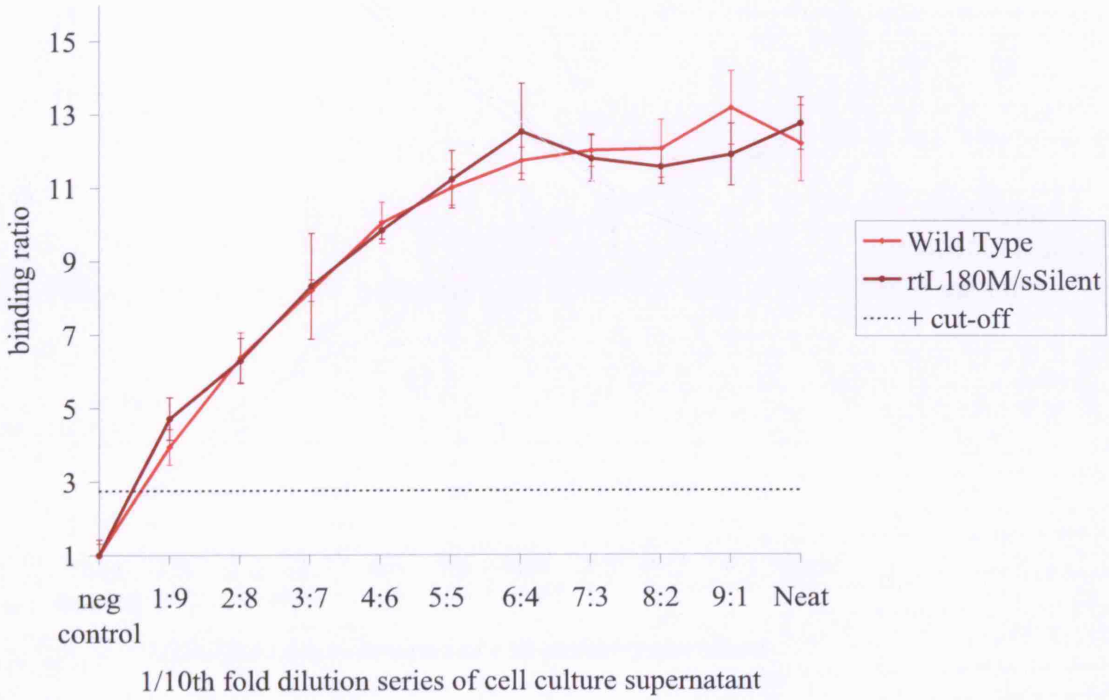


Figure A1.21 – Mean binding ratios of rtL180M/sSilent mutant HBsAg in monoclonal D2H5 capture ELISA.

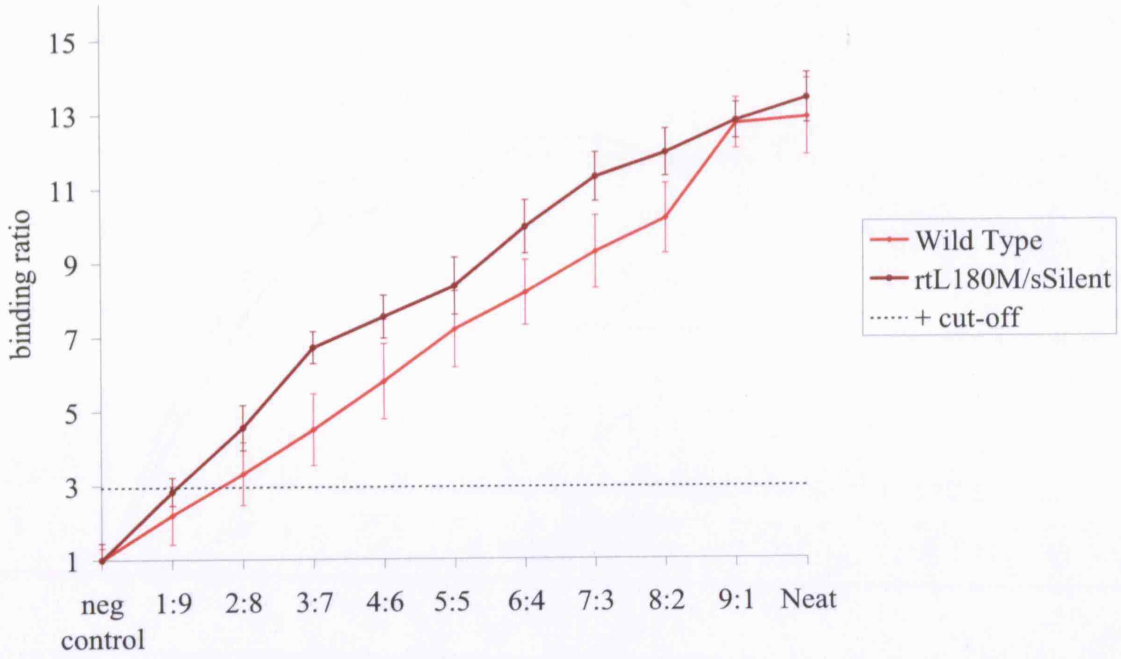


Figure A1.22 – Mean binding ratios of rtL180M/sSilent mutant HBsAg in monoclonal H3F5 capture ELISA.

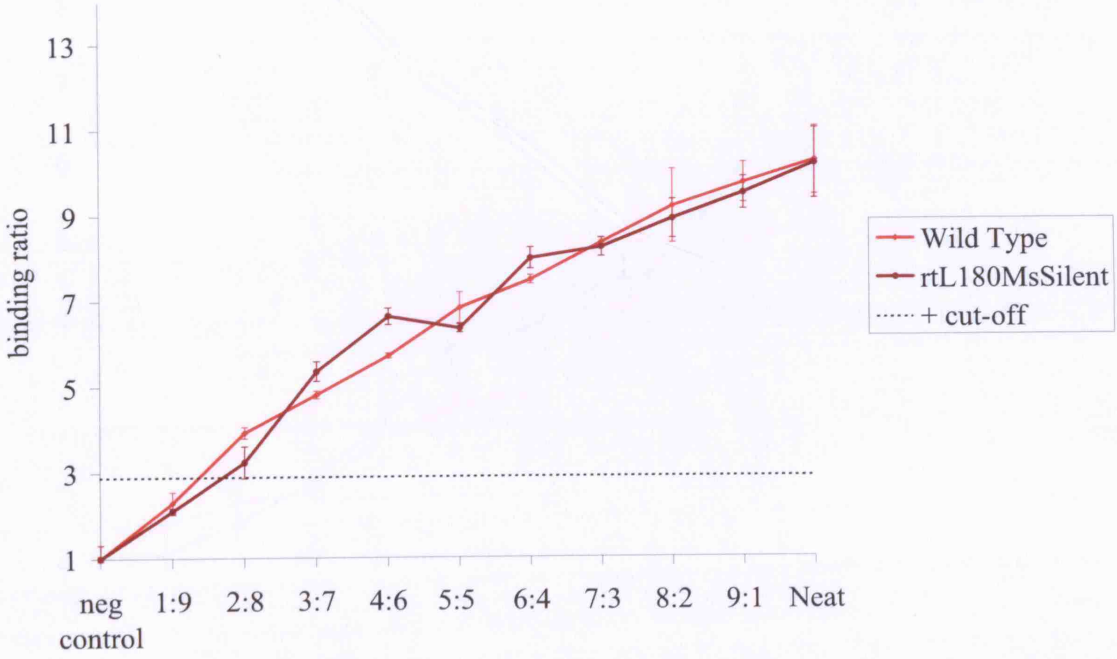


Figure A1.23 – Mean binding ratios of rtL180M/sSilent mutant HBsAg in Ge34/36 format capture ELISA.

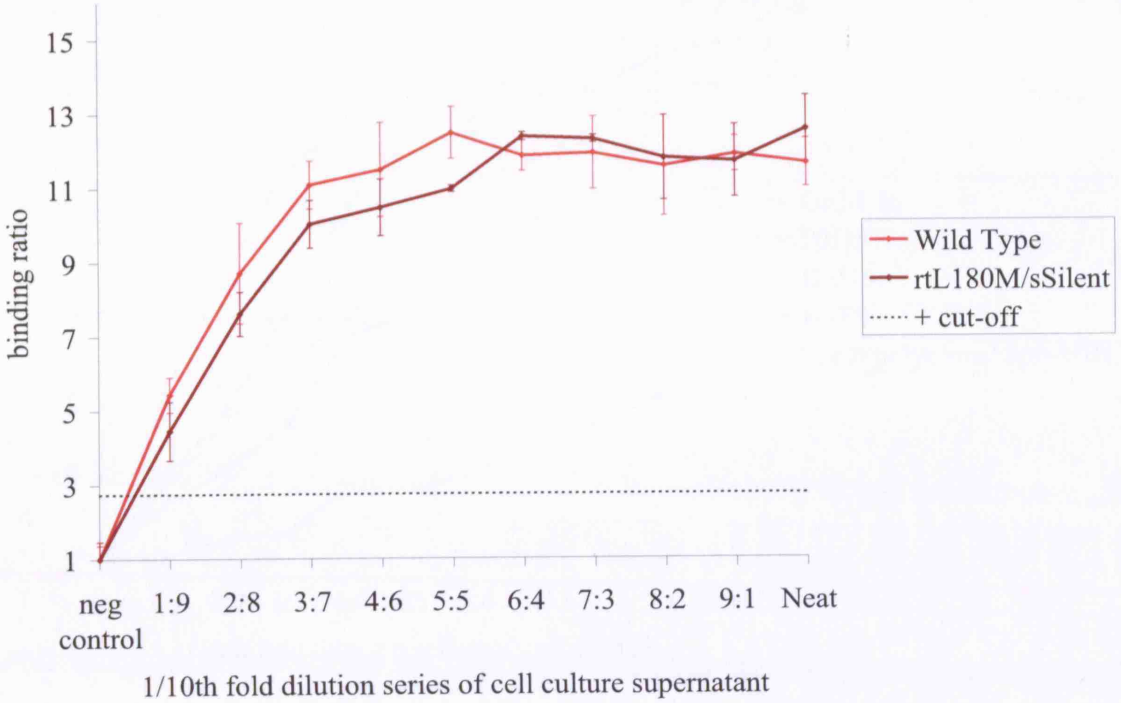


Figure A1.24 – Mean binding ratios of rtL180M/sSilent mutant HBsAg in horse polyclonal anti-HBs capture ELISA.

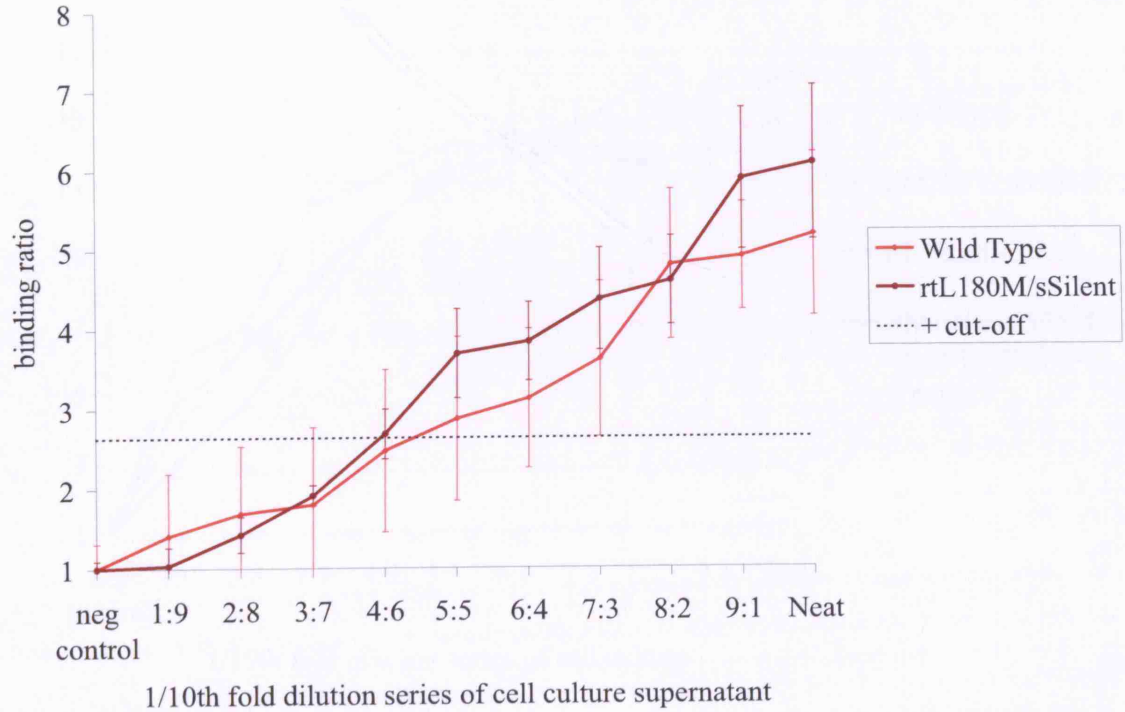


Figure A1.25 – Mean binding ratios of rtL180M/sSilent + rtM204V/sI195M mutant HBsAg in single monoclonal, multiple monoclonal and polyclonal capture ELISAs.

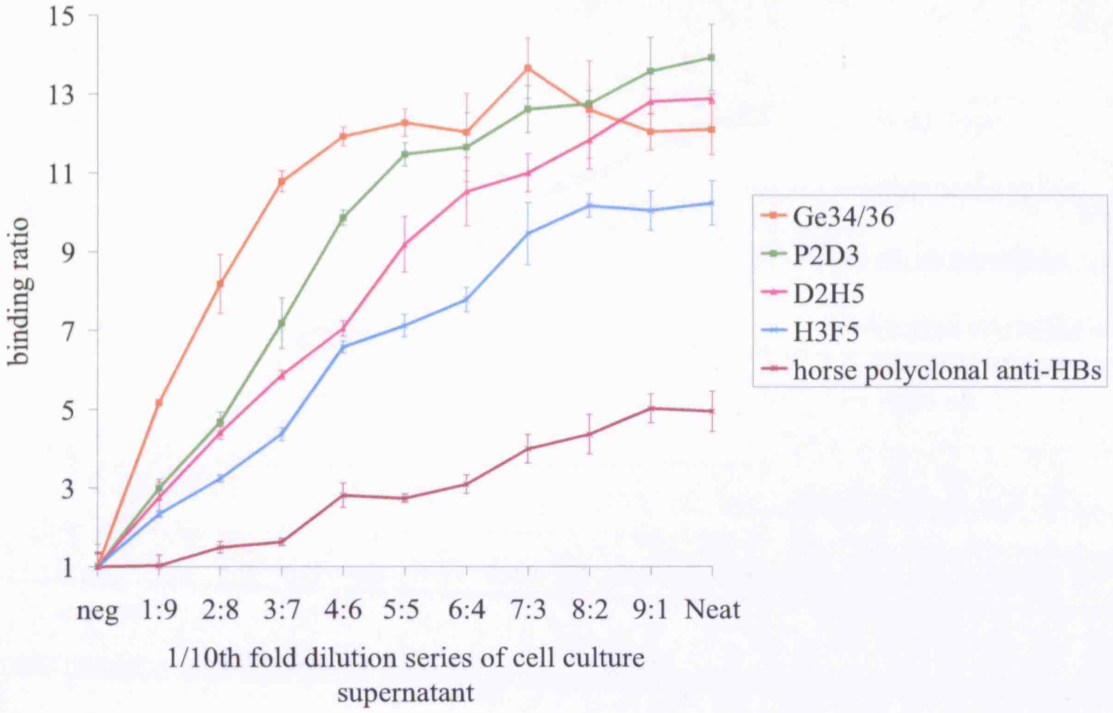


Figure A1.26 – Mean binding ratios of rtL180M/sSilent + rtM204V/sI195M mutant HBsAg in monoclonal P2D3 capture ELISA.

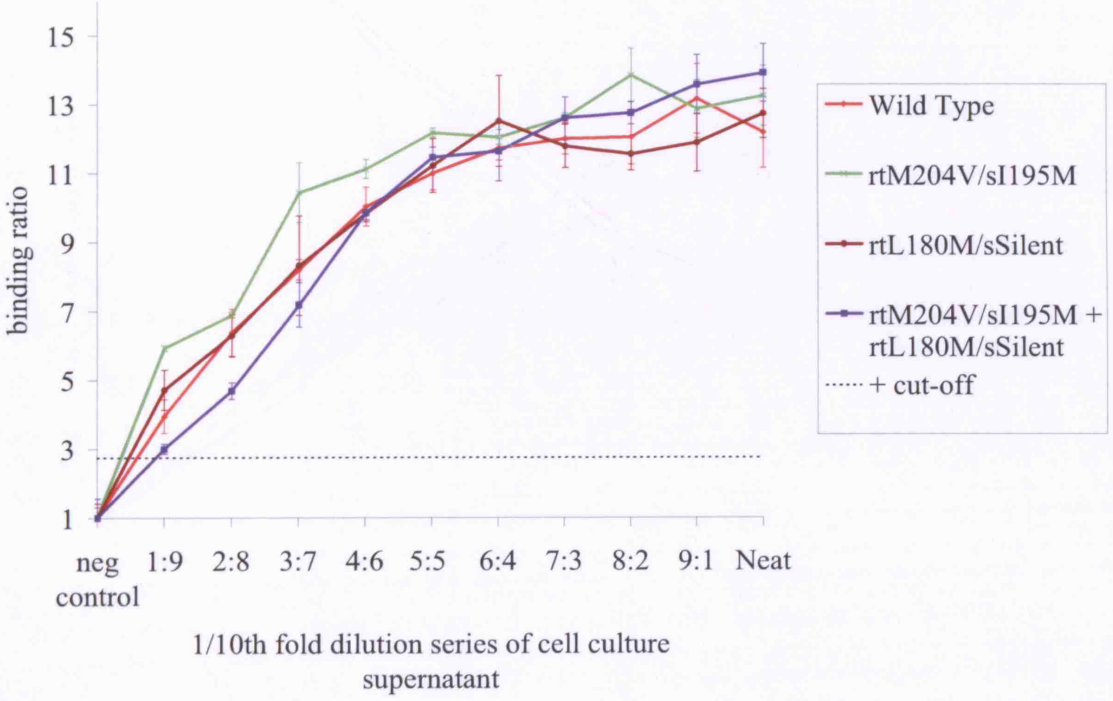


Figure A1.27 – Mean binding ratios of rtL180M/sSilent +rtM204V/sI195M mutant HBsAg in monoclonal D2H5 capture ELISA.

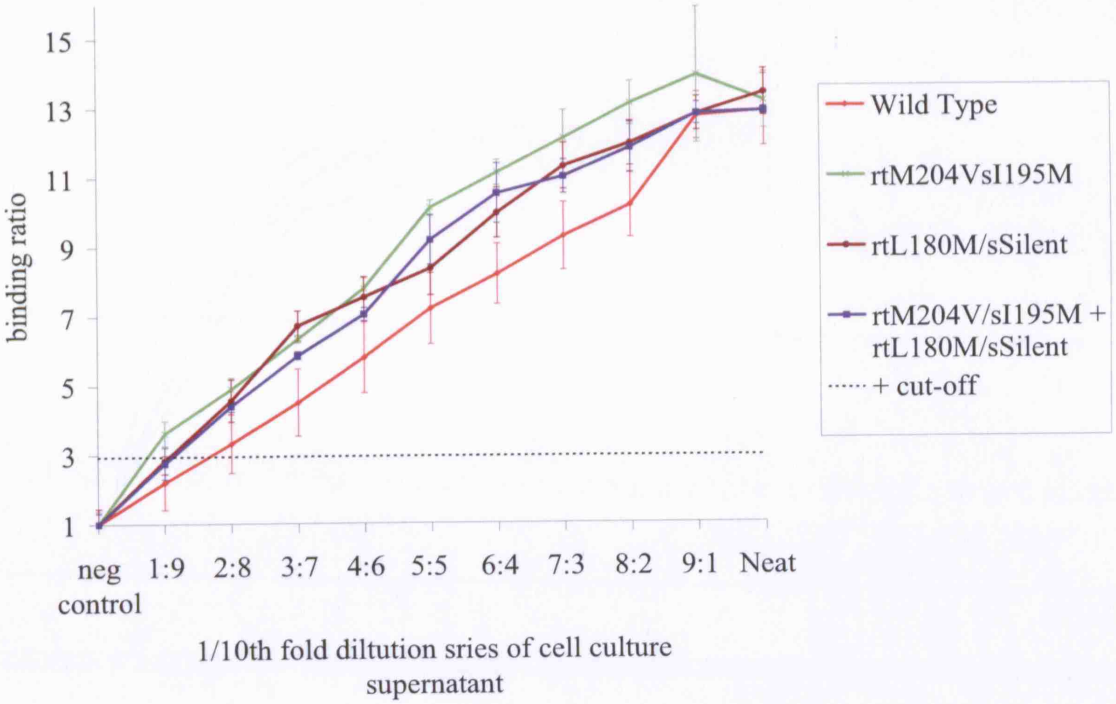


Figure A1.28 – Mean binding ratios of rtL180M/sSilent +rtM204V/sI195M mutant HBsAg in monoclonal H3F5 capture ELISA.

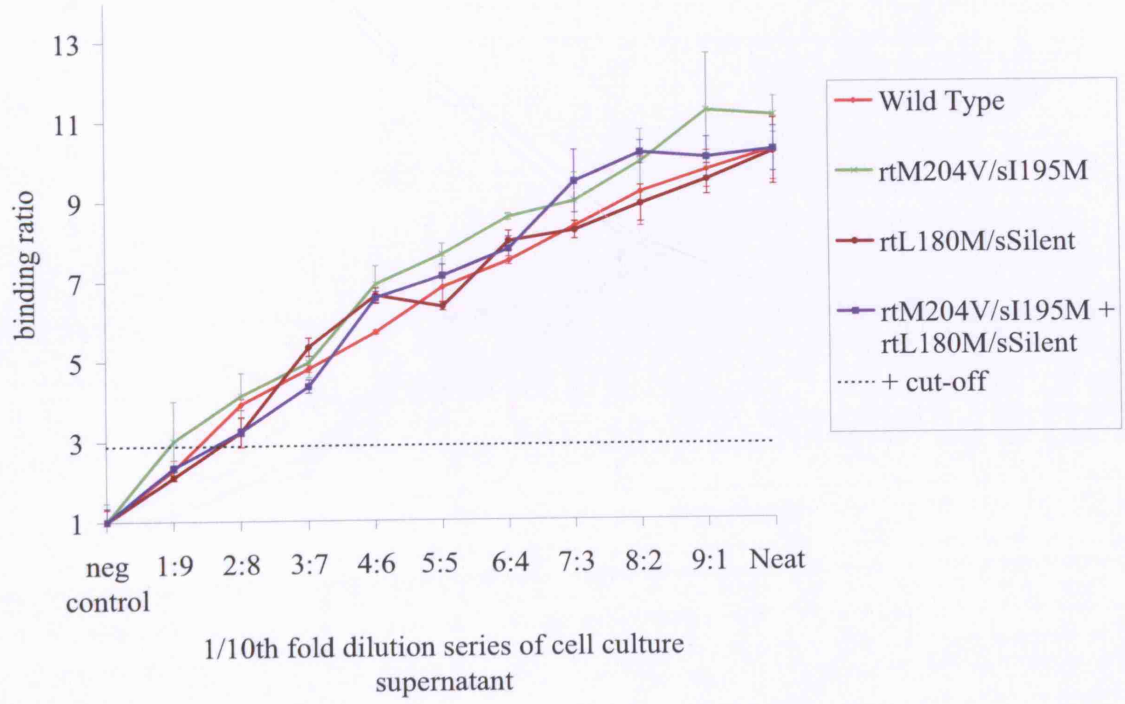


Figure A1.29 – Mean binding ratios of rtL180M/sSilent + rtM204V/sI195M mutant HBsAg in Ge34/36 format capture ELISA.

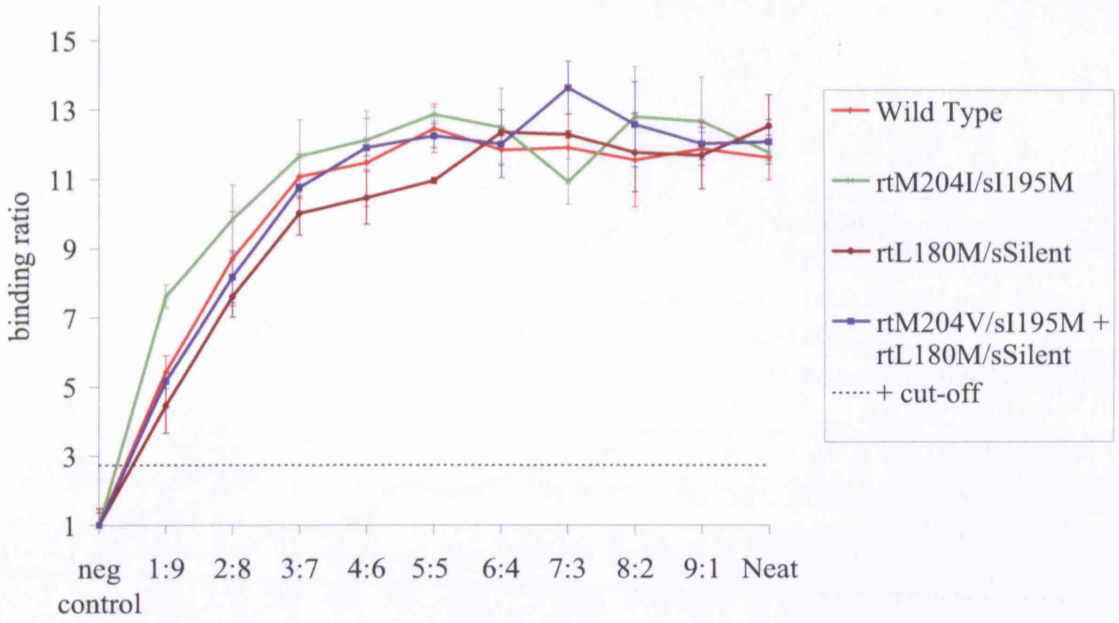


Figure A1.30 – Mean binding ratios of rtL180M/sSilent +rtM204V/sI195M mutant HBsAg in horse polyclonal anti-HBs capture ELISA.

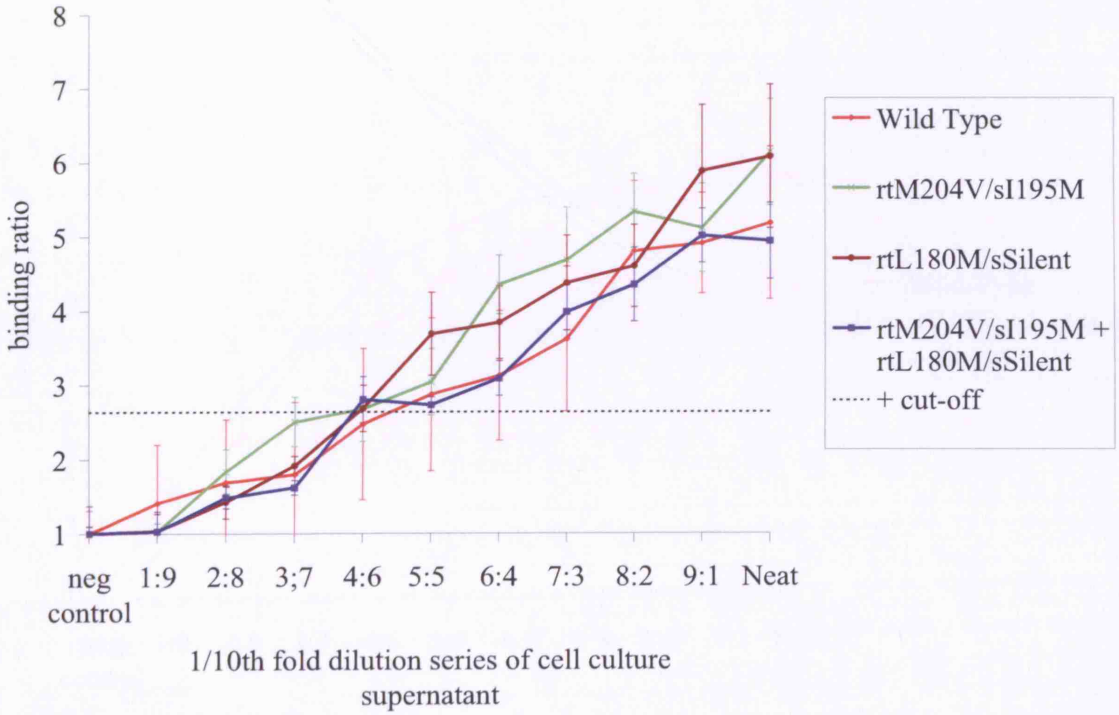


Figure A1.31 – Mean binding ratios of rtV173L/sE164D mutant HBsAg in single monoclonal, multiple monoclonal and polyclonal capture ELISAs.

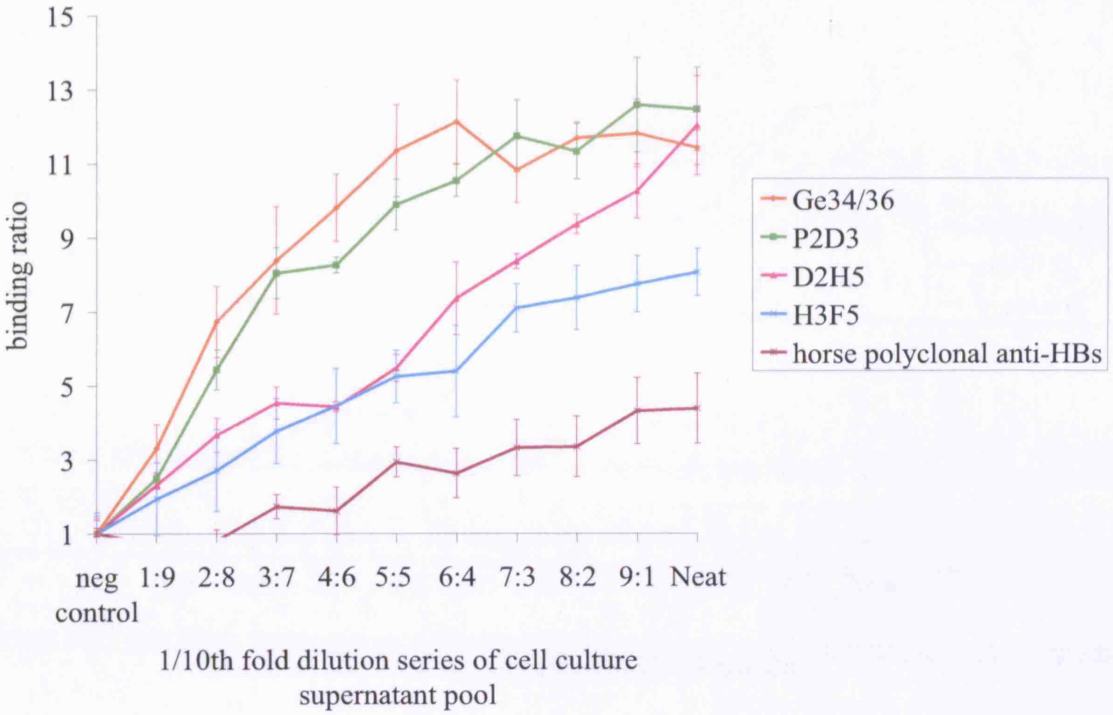


Figure A1.32 – Mean binding ratios of rtV173L/sE164D mutant HBsAg in monoclonal P2D3 capture ELISA.

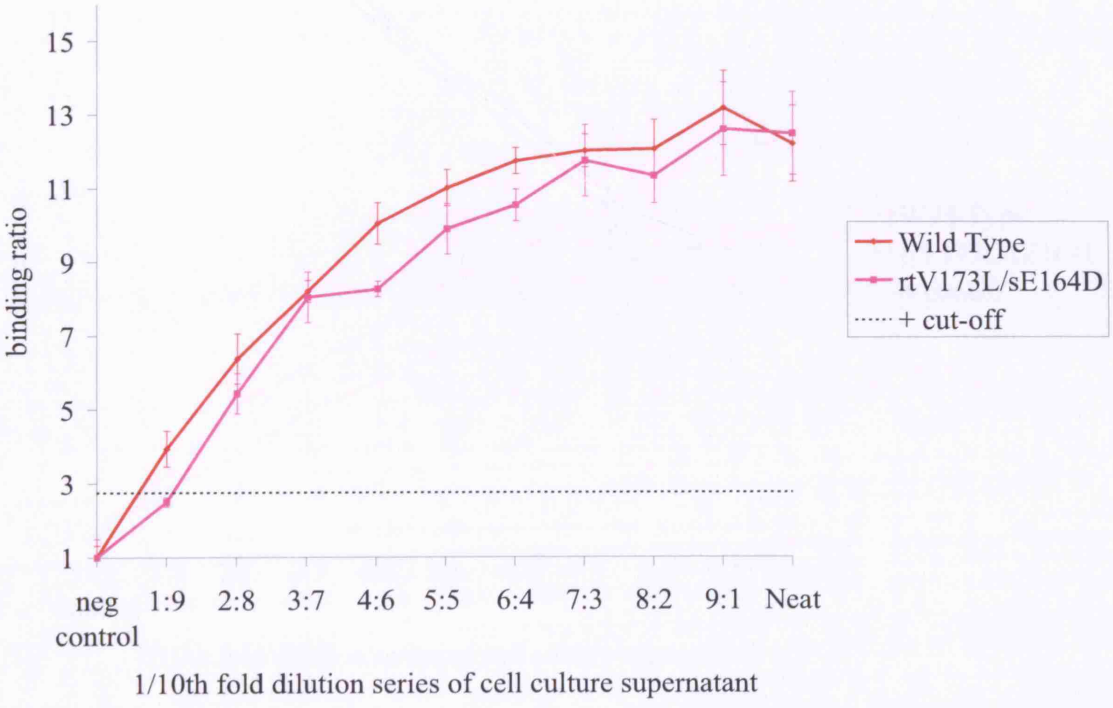


Figure A1.33 – Mean binding ratios of rtV173L/sE164D mutant HBsAg in monoclonal D2H5 capture ELISA.

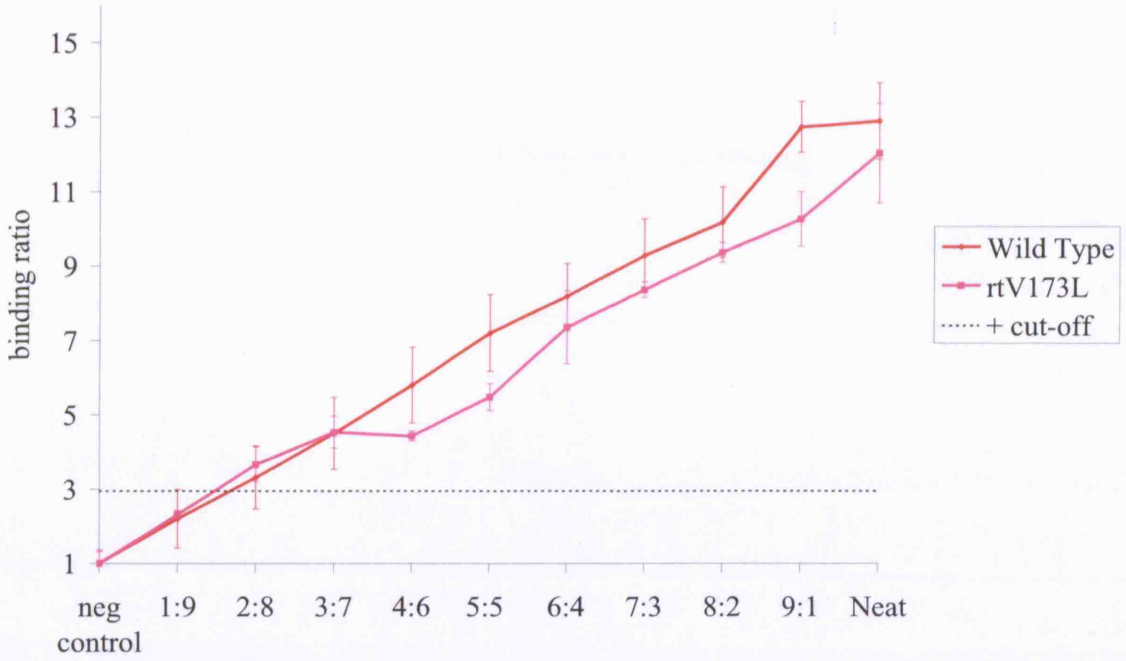


Figure A1.34 – Mean binding ratios of rtV173L/sE164D mutant HBsAg in monoclonal H3F5 capture ELISA.

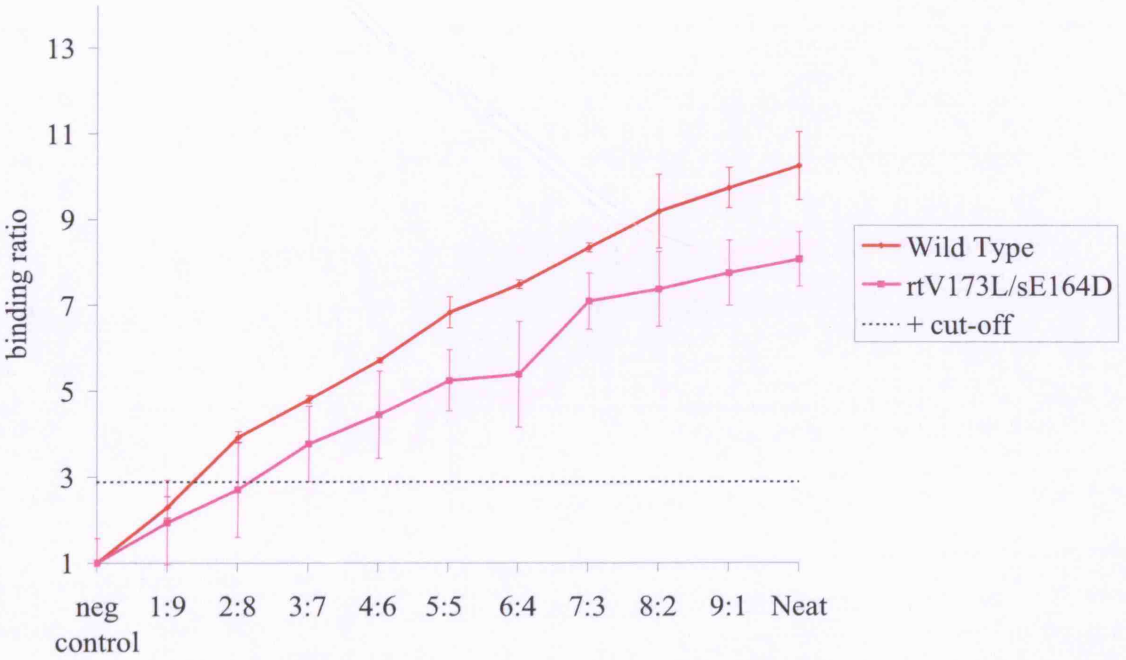


Figure A1.35 – Mean binding ratios of rtV173L/sE164D mutant HBsAg in Ge34/36 format capture ELISA.

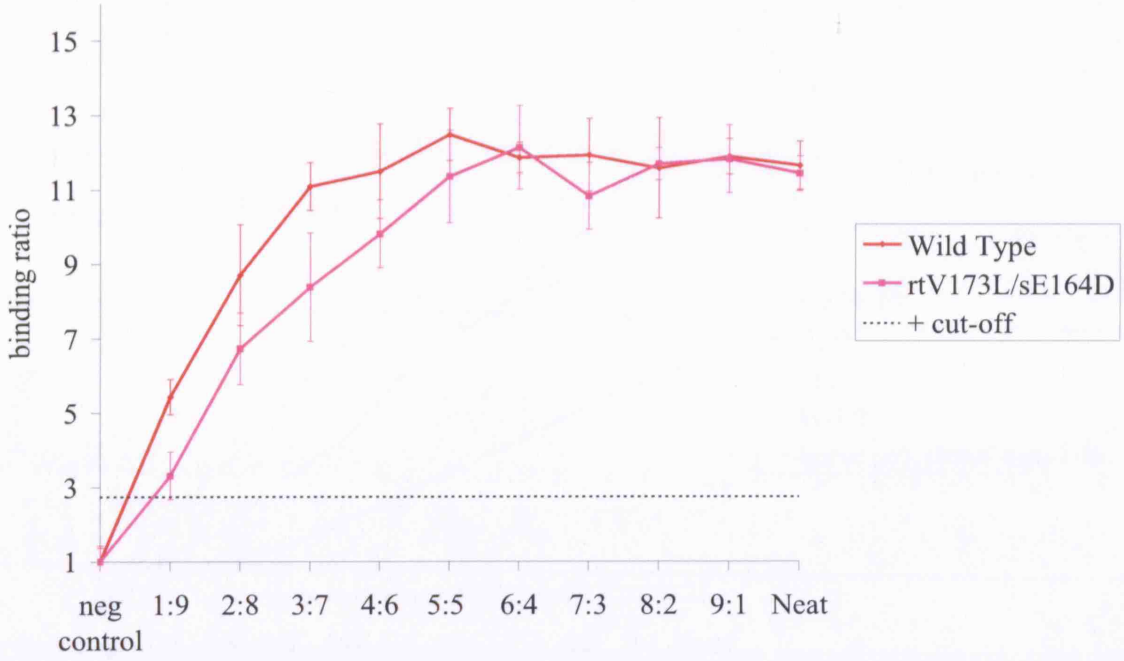


Figure A1.36 – Mean binding ratios of rtV173L/sE164D mutant HBsAg in horse polyclonal anti-HBs capture ELISA.

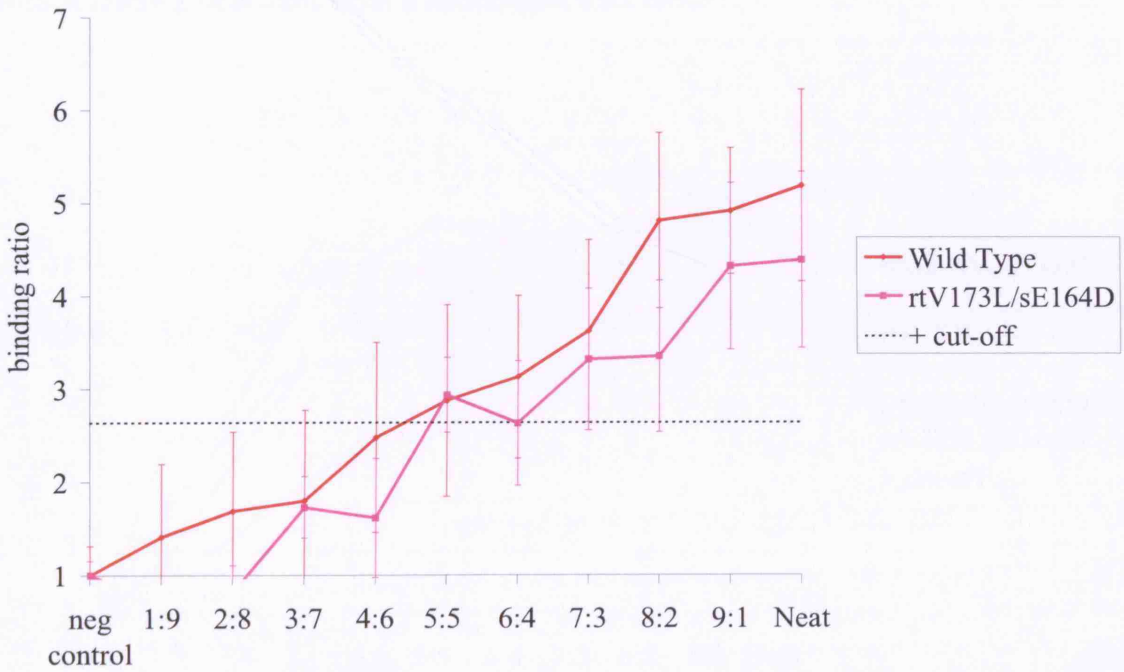


Figure A1.37 – Mean binding ratios of rtV173L/sE164D + rtM204V/sI195M mutant HBsAg in single monoclonal, multiple monoclonal and polyclonal capture ELISAs.

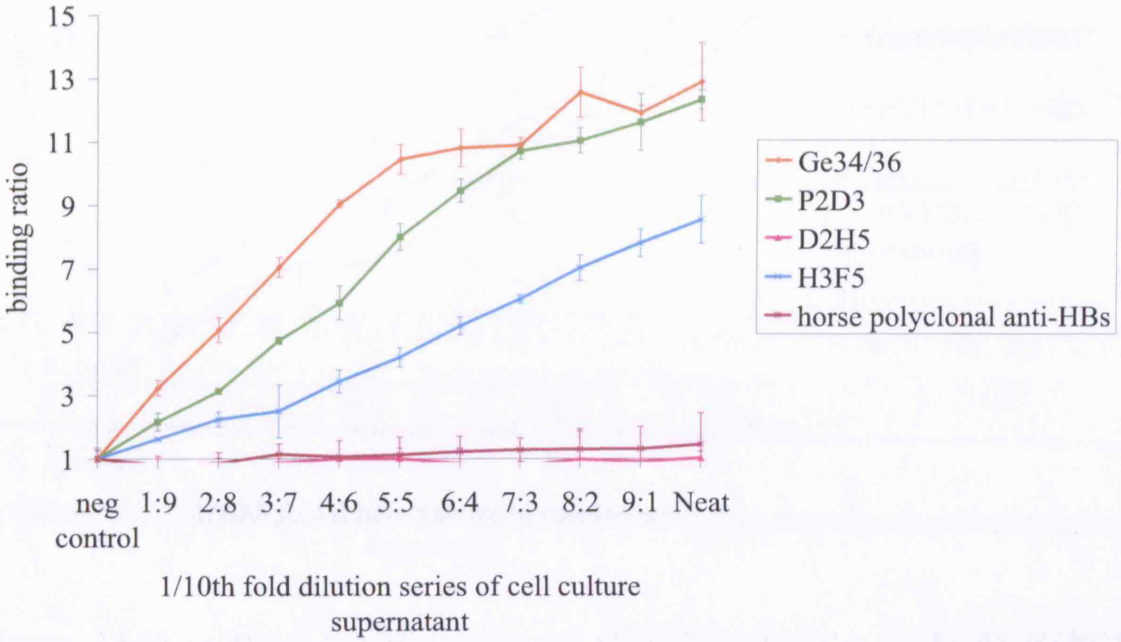


Figure A1.38 – Mean binding ratios of rtV173L/sE164D + rtM204V/sI195M mutant HBsAg in monoclonal P2D3 capture ELISA.

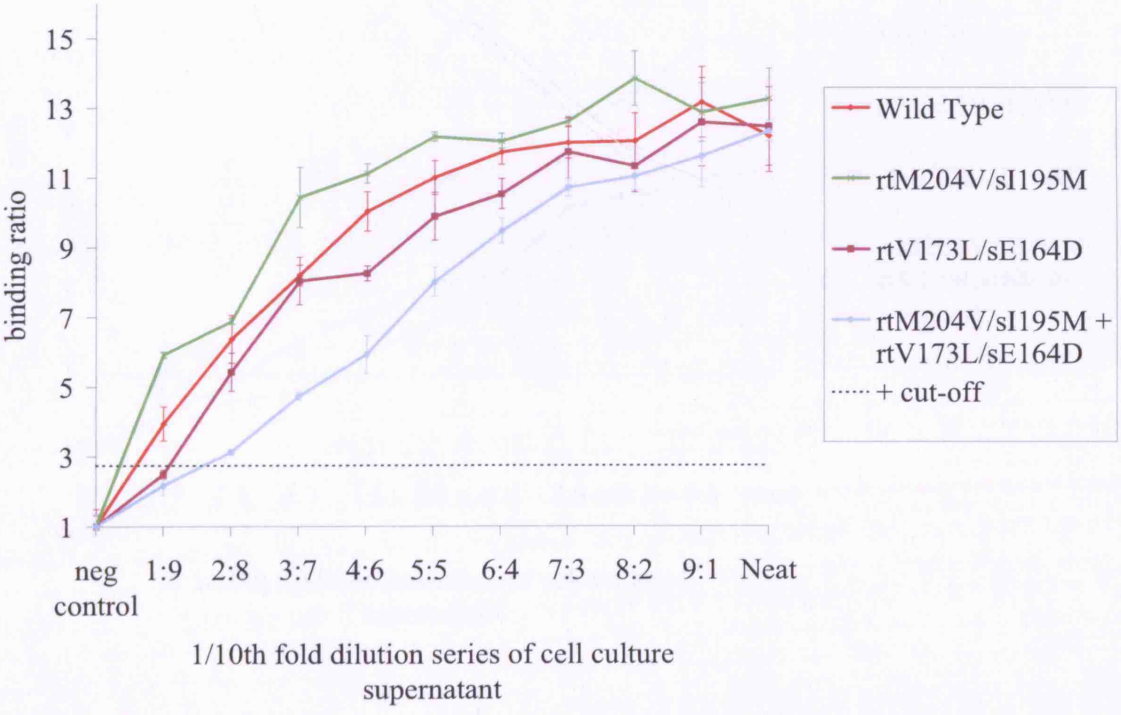


Figure A1.39 – Mean binding ratios of rtV173L/sE164D + rtM204V/sI195M mutant HBsAg in monoclonal D2H5 capture ELISA.

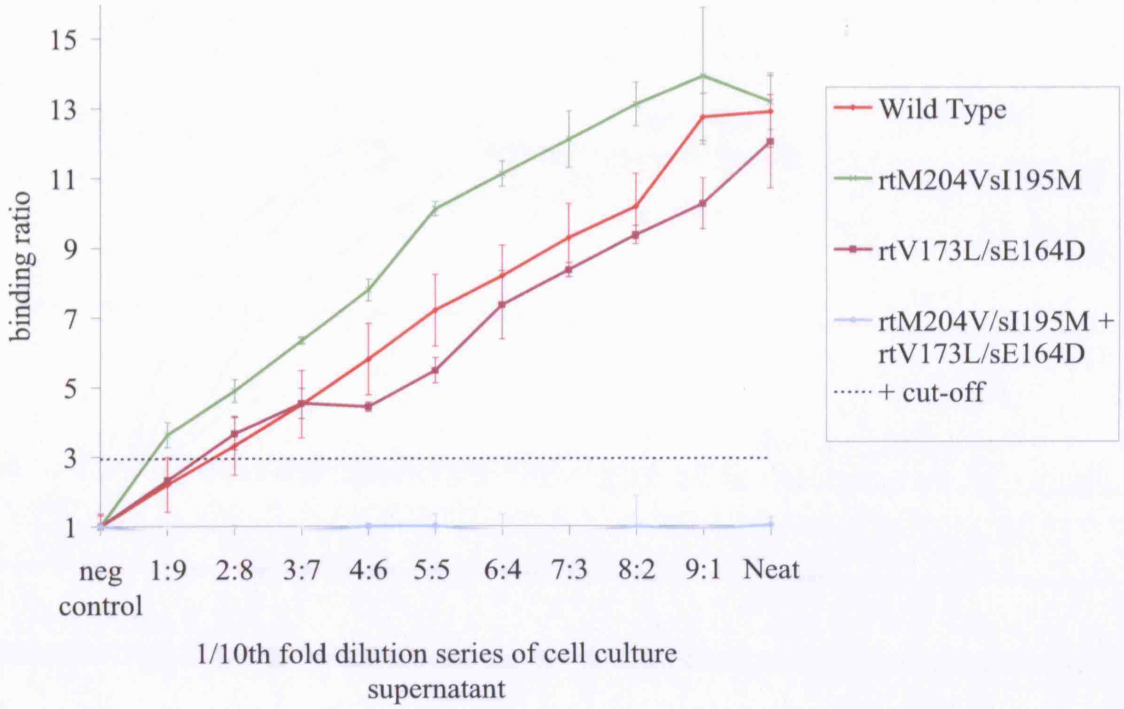


Figure A1.40 – Mean binding ratios of rtV173L/sE164D + rtM204V/sI195M mutant HBsAg in monoclonal H3F5 capture ELISA.

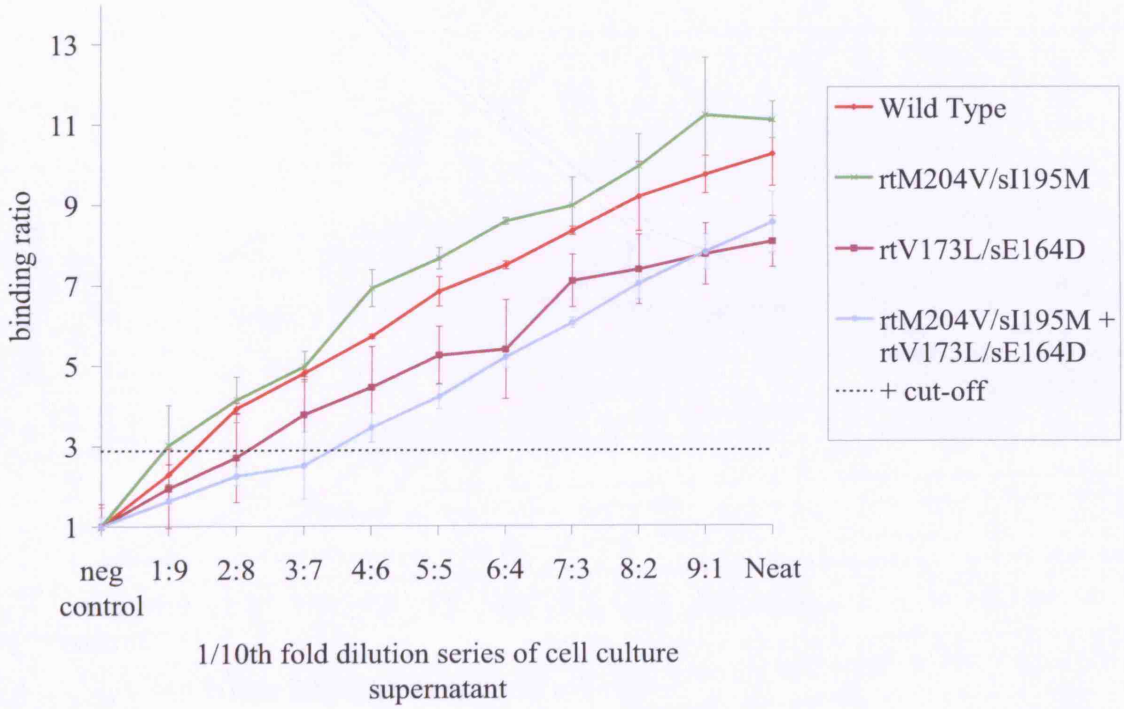


Figure A1.41 – Mean binding ratios of rtV173L/sE164D + rtM204V/sI195M mutant HBsAg in Ge34/36 format capture ELISA.

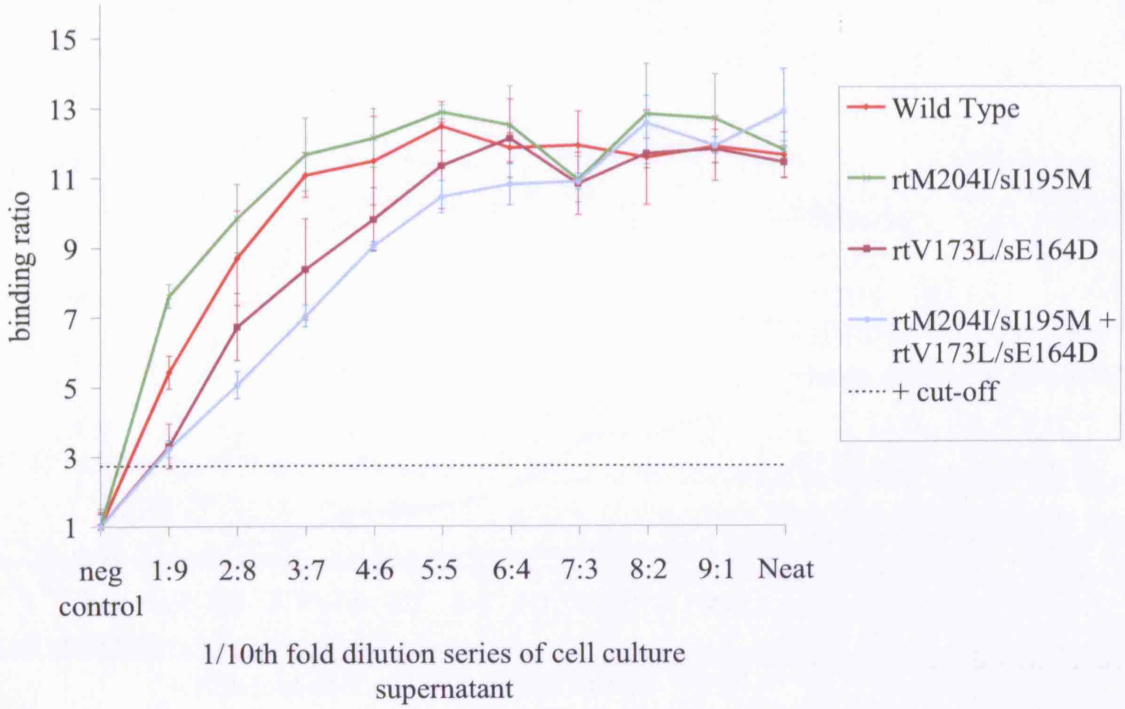


Figure A1.42 – Mean binding ratios of rtV173L/sE164D + rtM204V/sI195M mutant HBsAg in horse polyclonal anti-HBs capture ELISA.

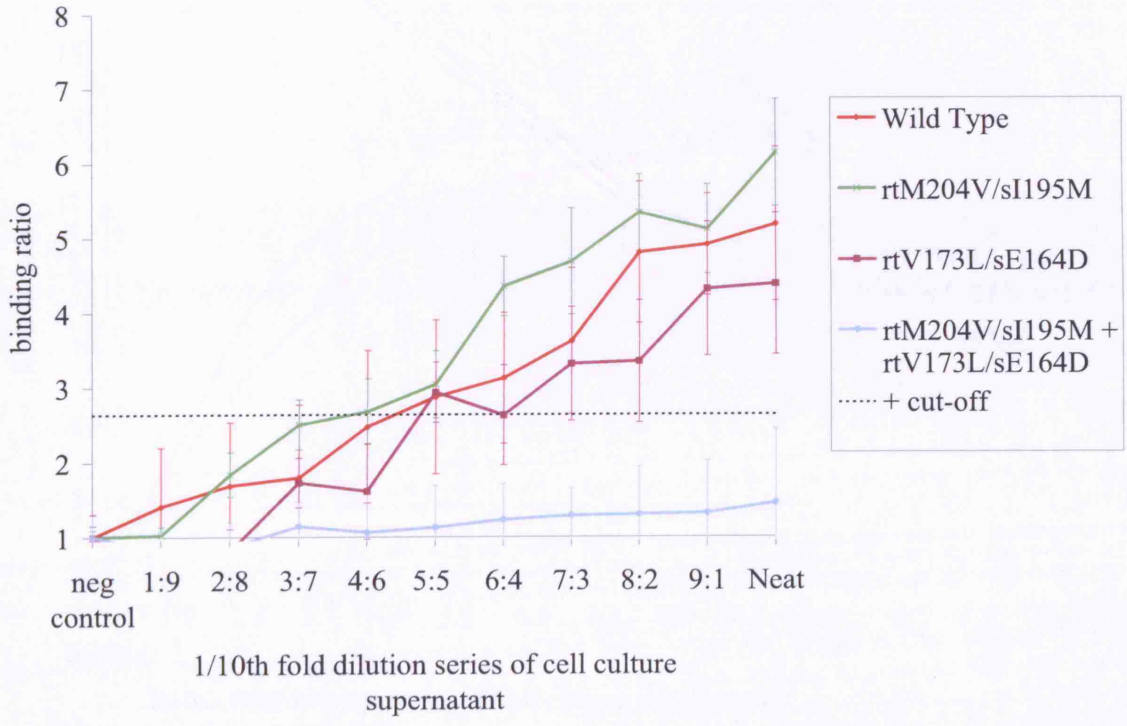


Figure A1.43 – Mean binding ratios of rtT128N/sP120T mutant HBsAg in single monoclonal, multiple monoclonal and polyclonal capture ELISAs.

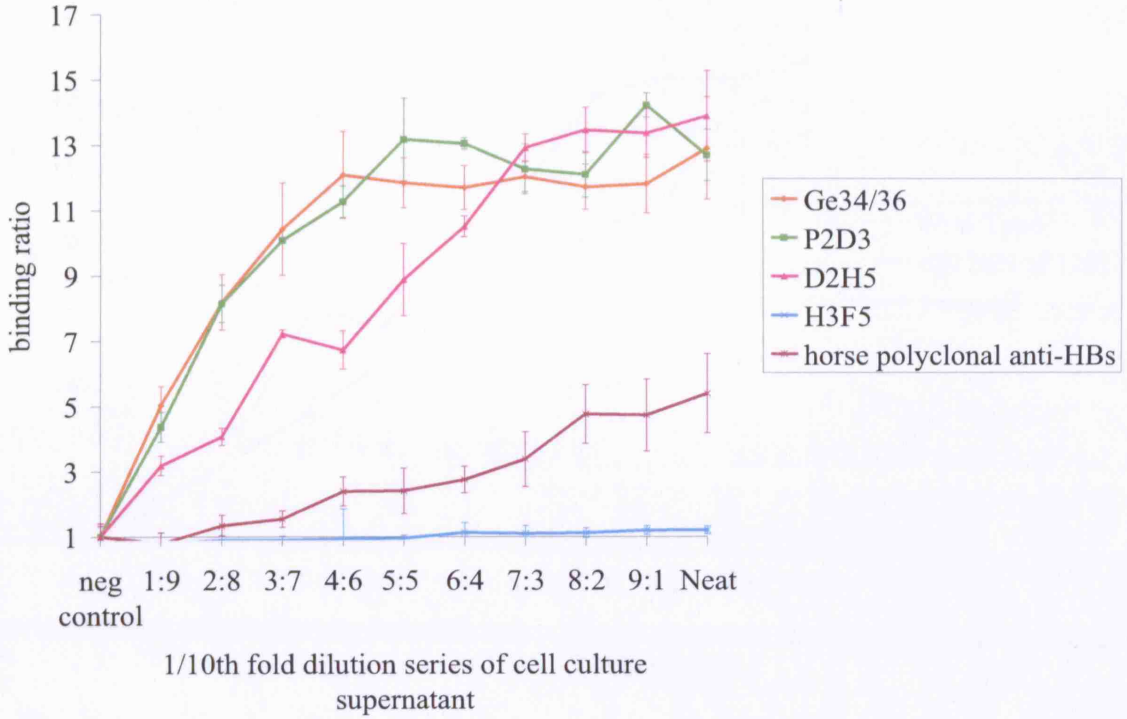


Figure A1.44 – Mean binding ratios of rtT128Ns/P120T mutant HBsAg in monoclonal P2D3 capture ELISA.

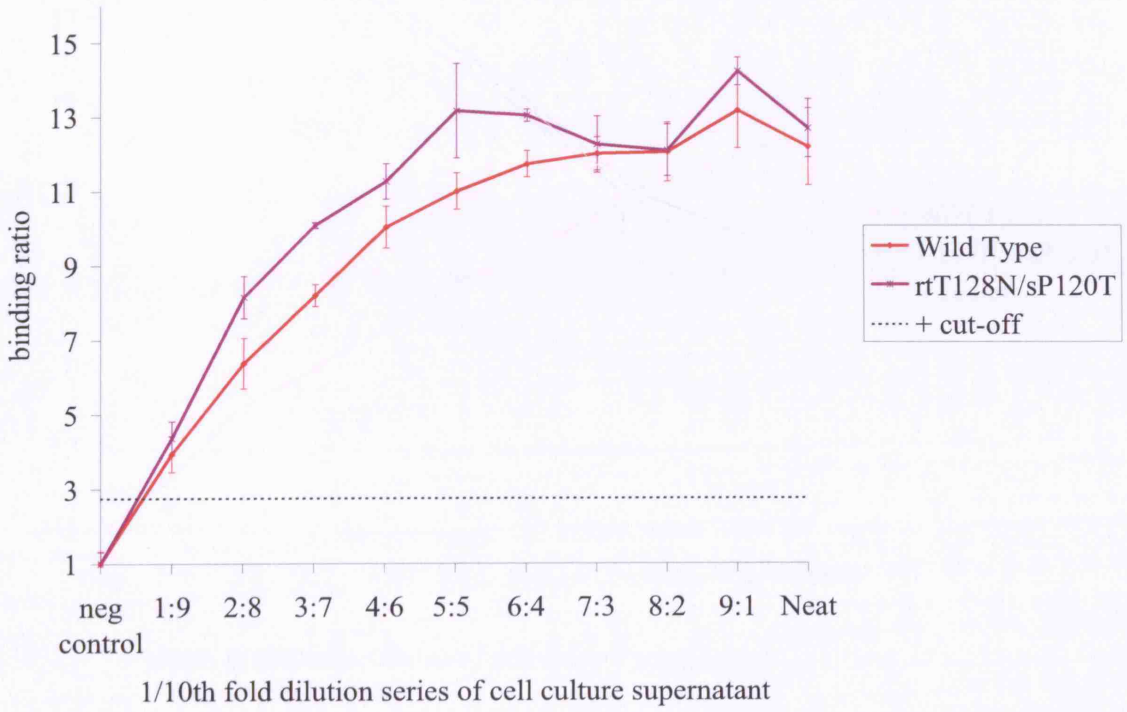


Figure A1.45 – Mean binding ratios of rtT128Ns/P120T mutant HBsAg in monoclonal D2H5 capture ELISA.

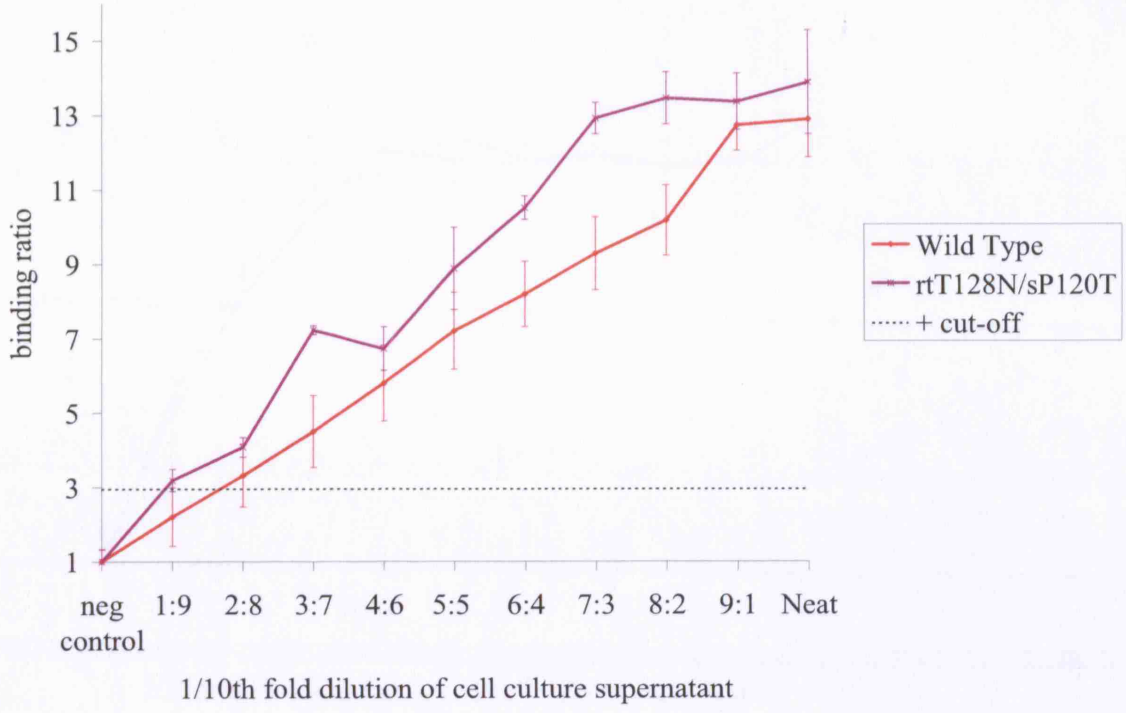


Figure A1.46 – Mean binding ratios of rtT128Ns/P120T mutant HBsAg in monoclonal H3F5 capture ELISA.

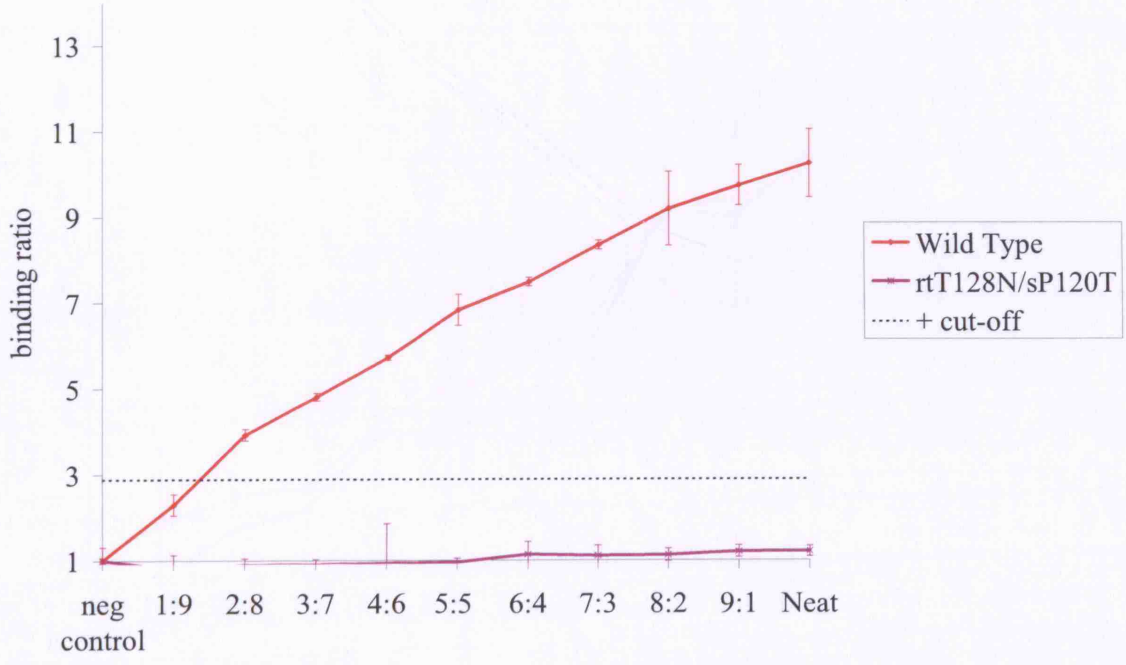


Figure A1.47 – Mean binding ratios of rtT128N/sP120T mutant HBsAg in Ge34/36 format capture ELISA.

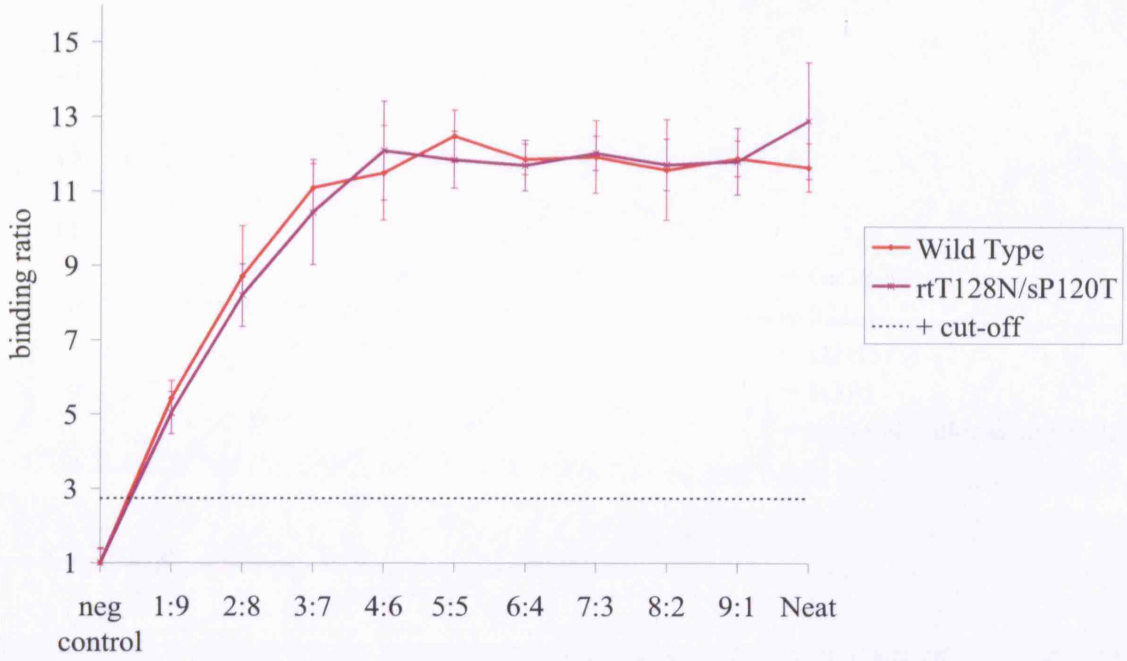


Figure A1.48 – Mean binding ratios of rtT128N/sP120T mutant HBsAg in horse polyclonal anti-HBs capture ELISA.

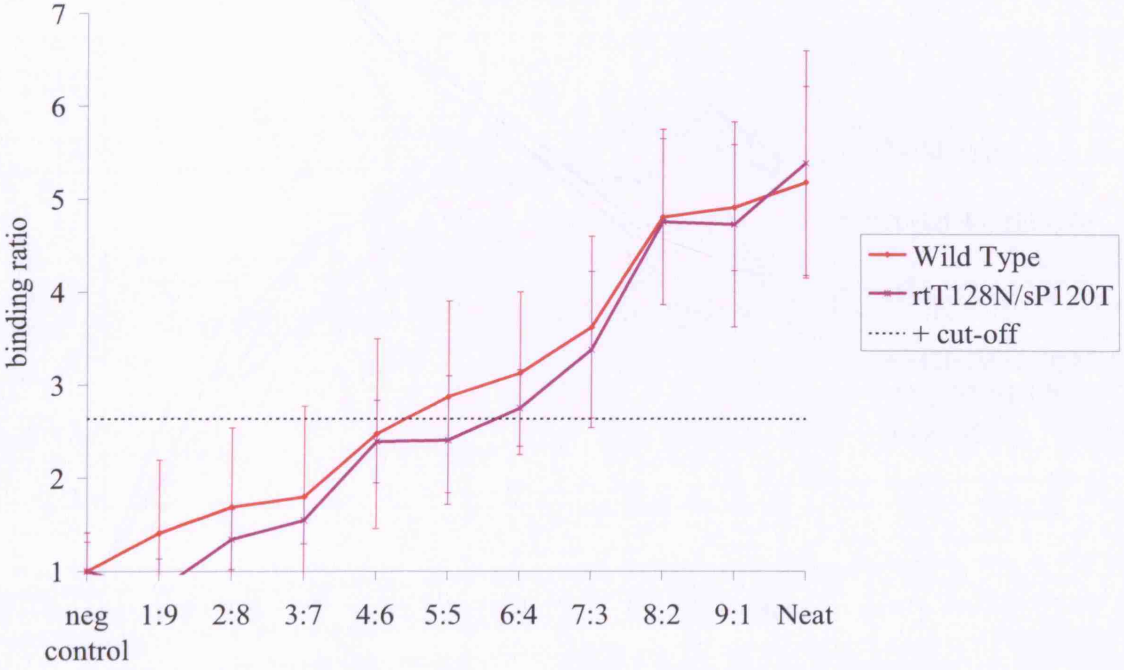


Figure A1.49 – Mean binding ratios of rtT128N/sP120T + rtM204V/sI195M mutant HBsAg in single monoclonal, multiple monoclonal and polyclonal capture ELISAs.

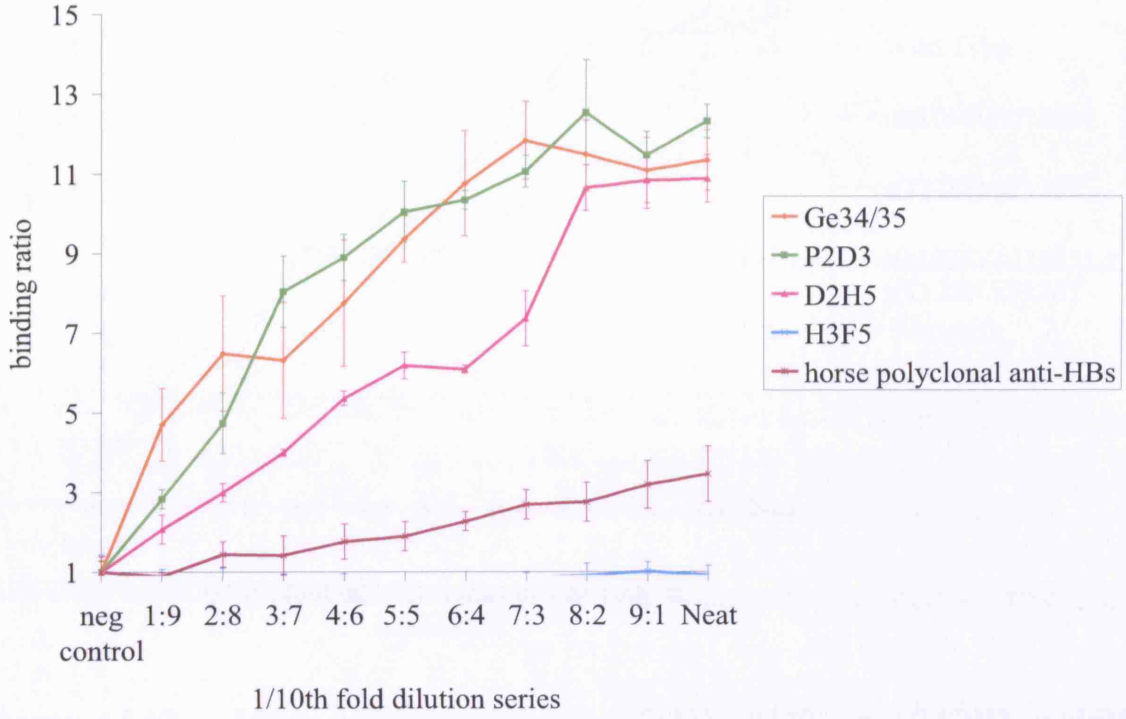


Figure A1.50 – Mean binding ratios of rtT128Ns/P120T + rtM204V/sI195M mutant HBsAg in monoclonal P2D3 capture ELISA.

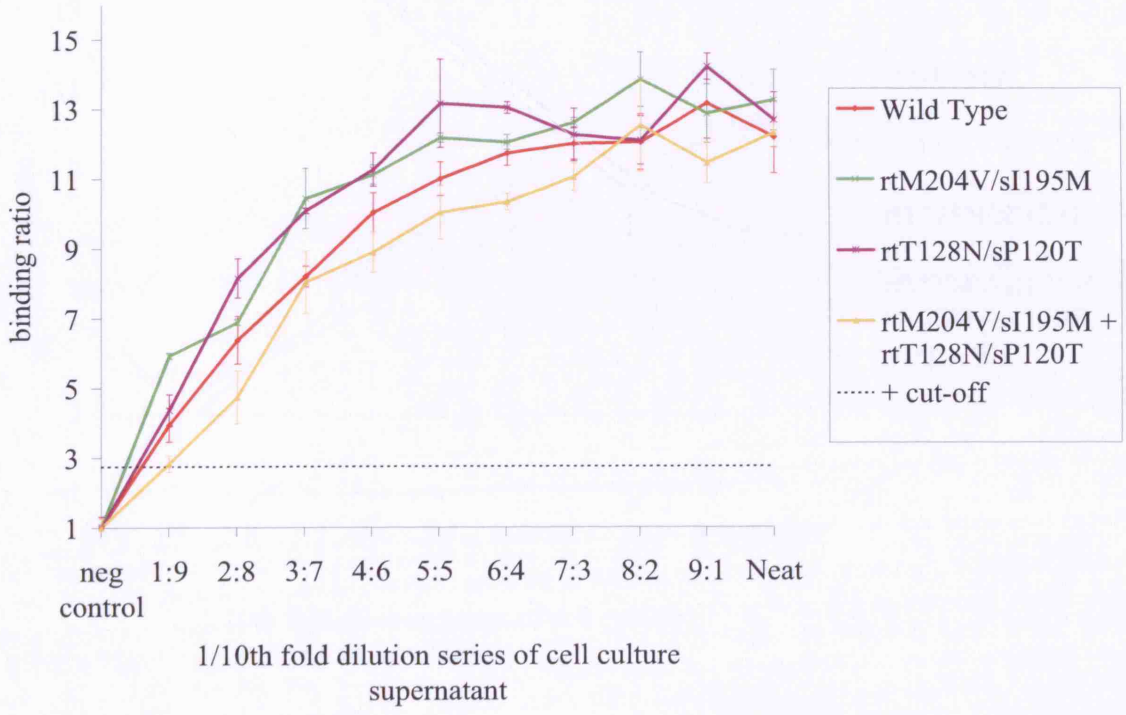


Figure A1.51 – Mean binding ratios of rtT128Ns/P120T + rtM204V/sI195M mutant HBsAg in monoclonal D2H5 capture ELISA.

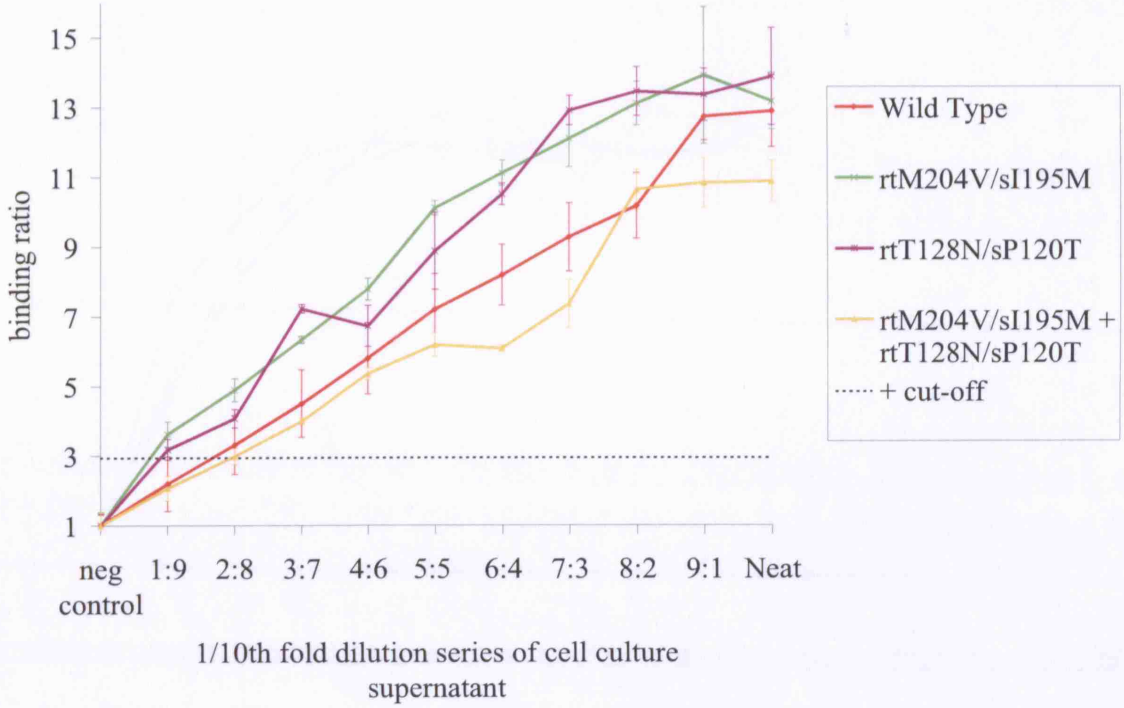


Figure A1.52 – Mean binding ratios of rtT128Ns/P120T + rtM204V/sI195M mutant HBsAg in monoclonal H3F5 capture ELISA.

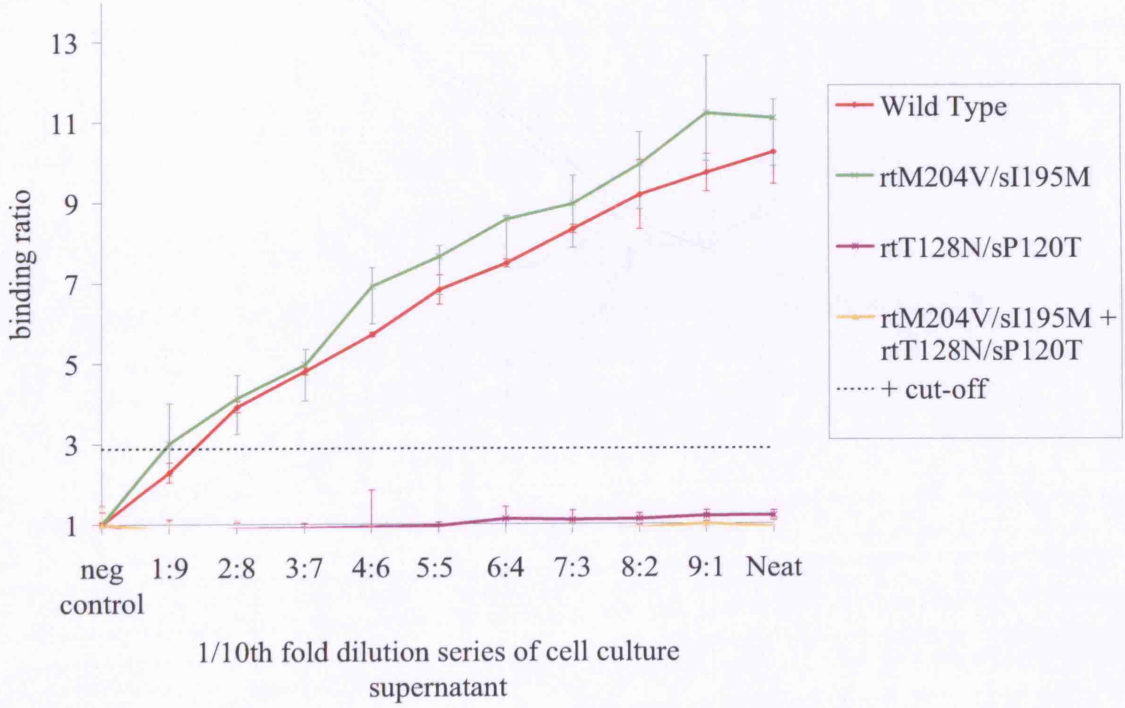


Figure A1.53 – Mean binding ratios of rtT128N/sP120T + rtM204V/sI195M mutant HBsAg in Ge34/36 format capture ELISA.

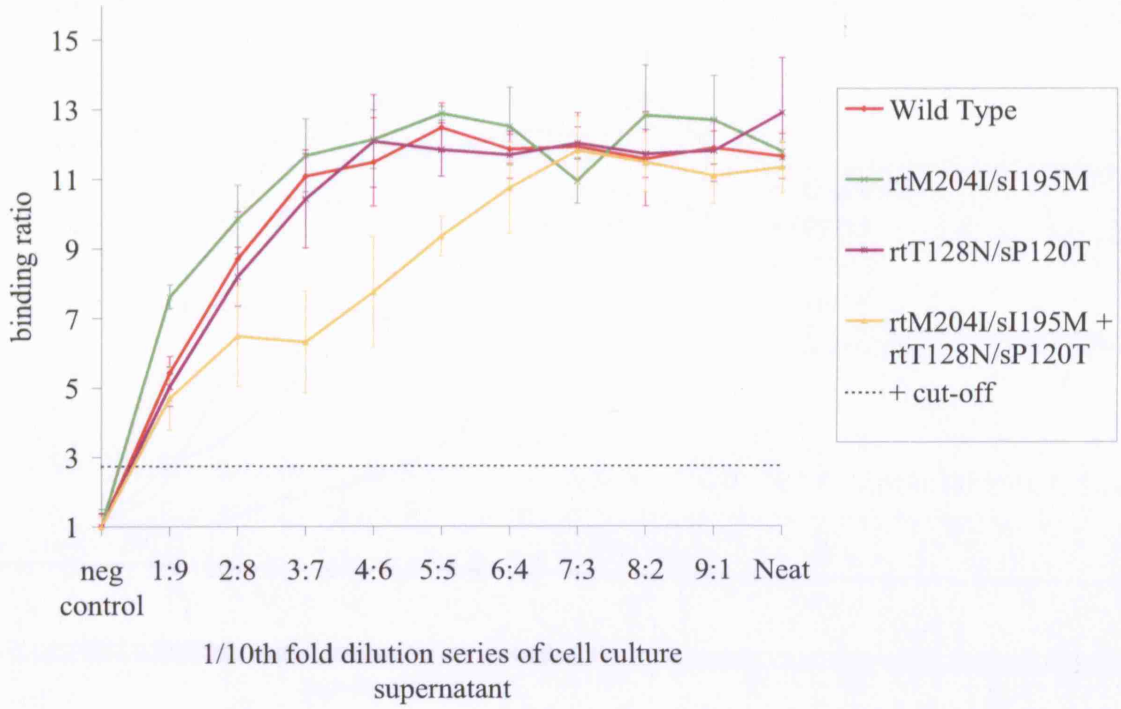


Figure A1.54 – Mean binding ratios of rtT128N/sP120T + rtM204V/sI195M mutant HBsAg in horse polyclonal anti-HBs capture ELISA.

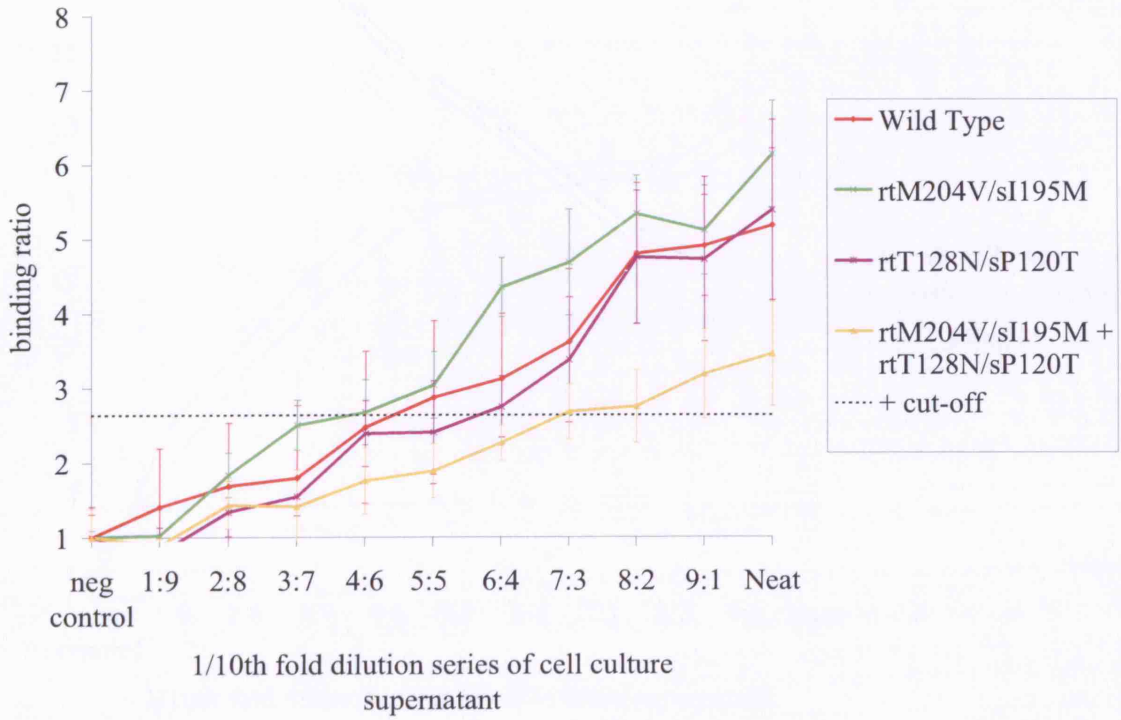


Figure A1.55 – Mean binding ratios of rtSilent/sD144E mutant HBsAg in single monoclonal, multiple monoclonal and polyclonal capture ELISAs.

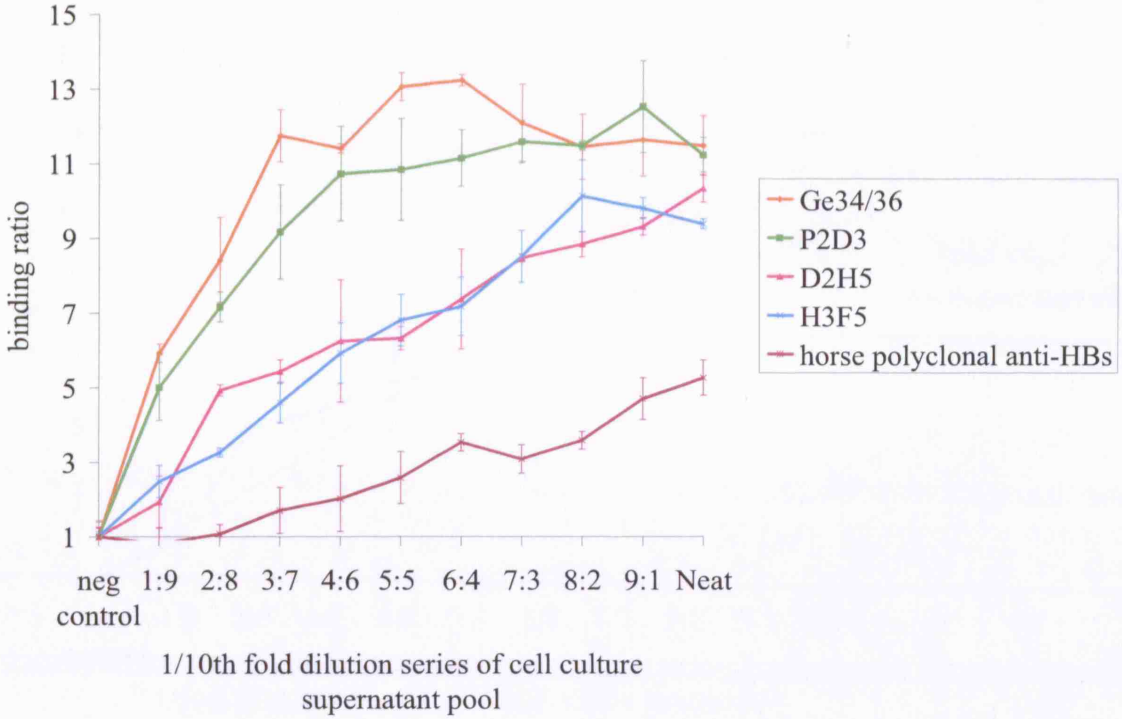


Figure A1.56 – Mean binding ratios of rtSilent/sD144E mutant HBsAg in monoclonal P2D3 capture ELISA.

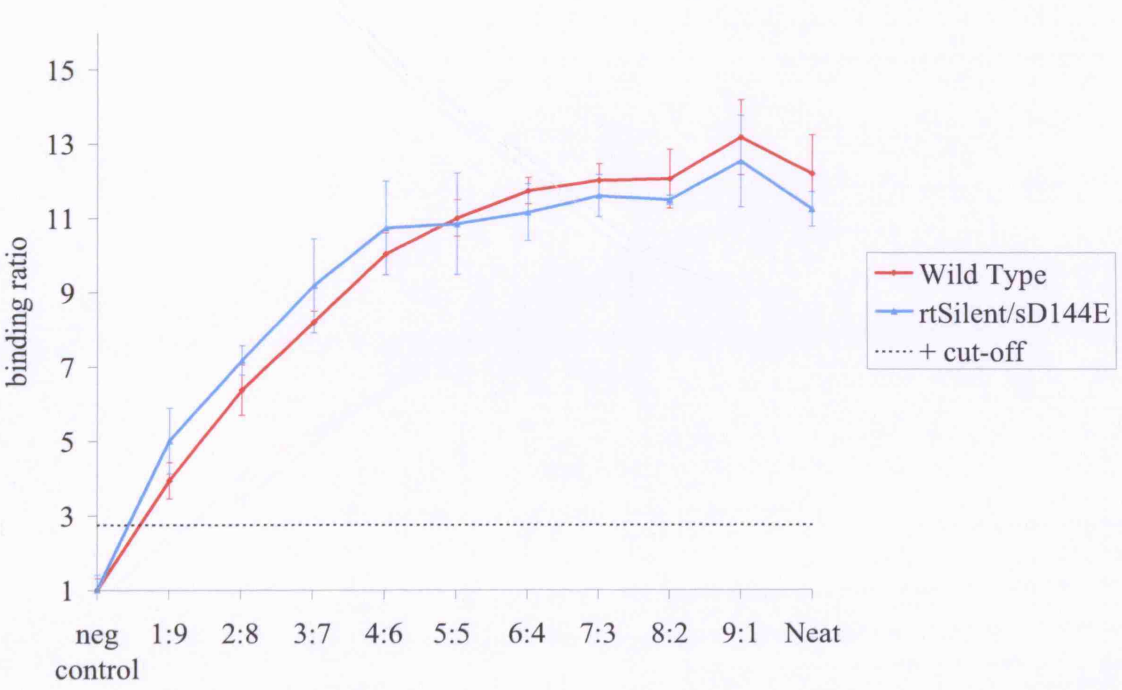


Figure A1.57 – Mean binding ratios of rtSilent/sD144E mutant HBsAg in monoclonal D2H5 capture ELISA.

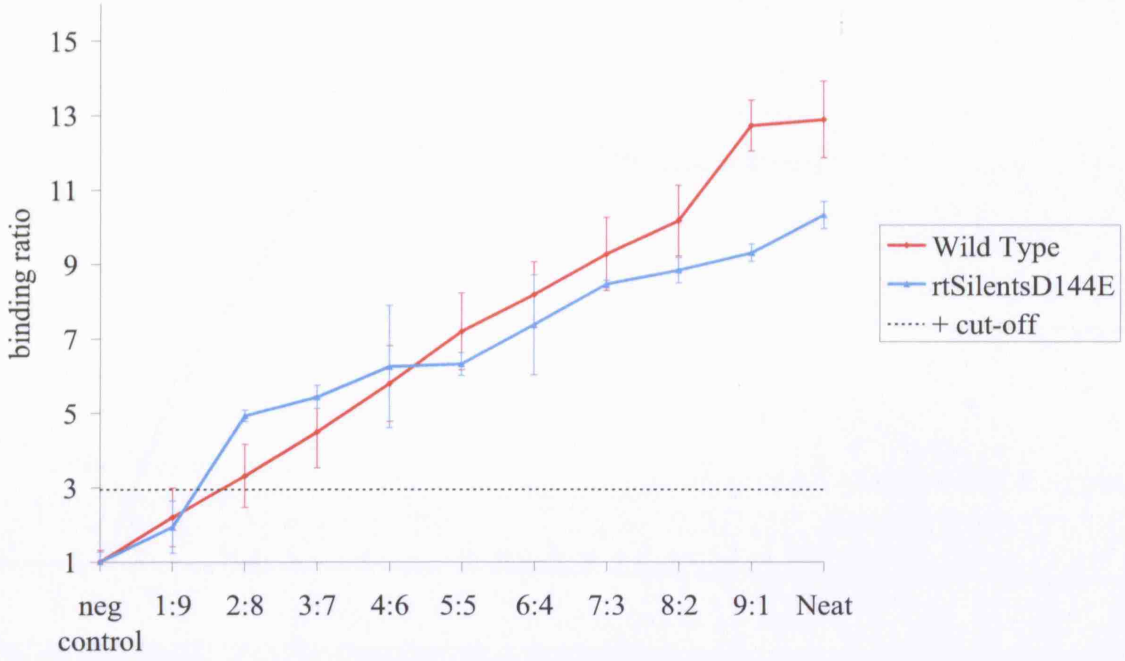


Figure A1.58 – Mean binding ratios of rtSilent/sD144E mutant HBsAg in monoclonal H3F5 capture ELISA.

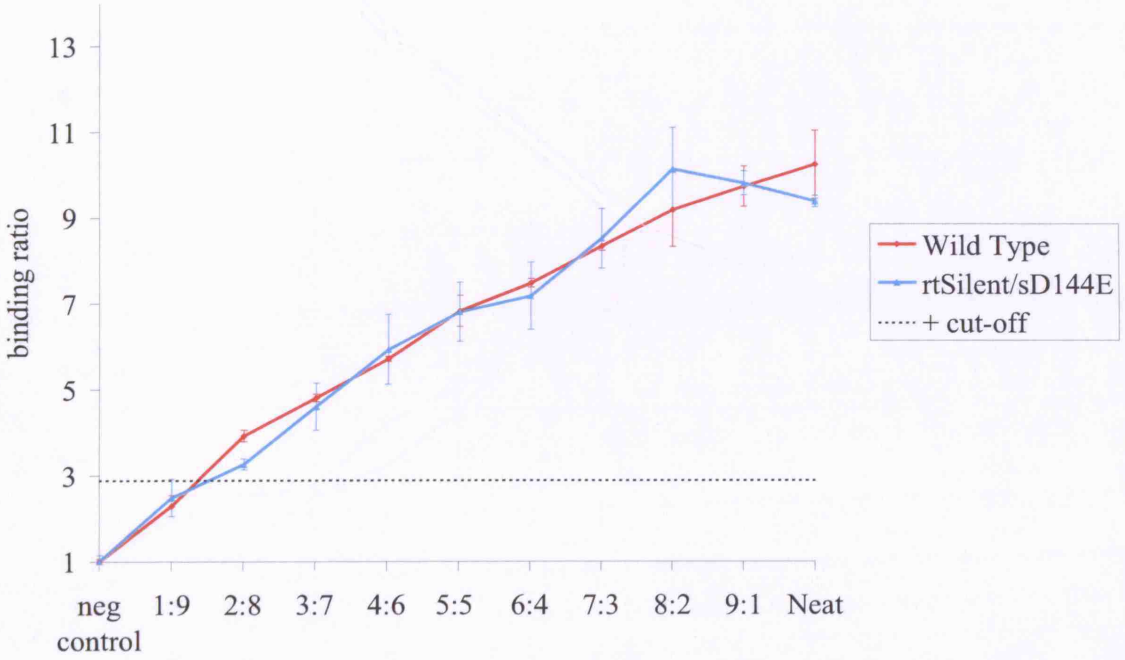


Figure A1.59 – Mean binding ratios of rtSilent/sD144E mutant HBsAg in Ge34/36 format capture ELISA.

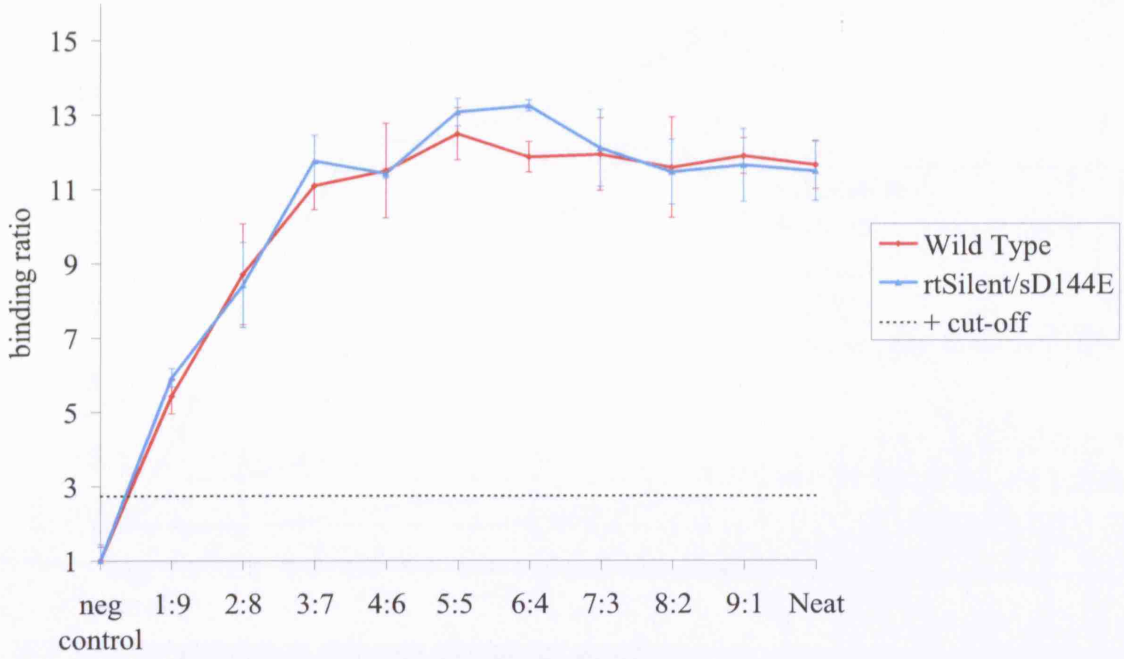


Figure A1.60 – Mean binding ratios of rtSilent/sD144E mutant HBsAg in horse polyclonal anti-HBs capture ELISA.

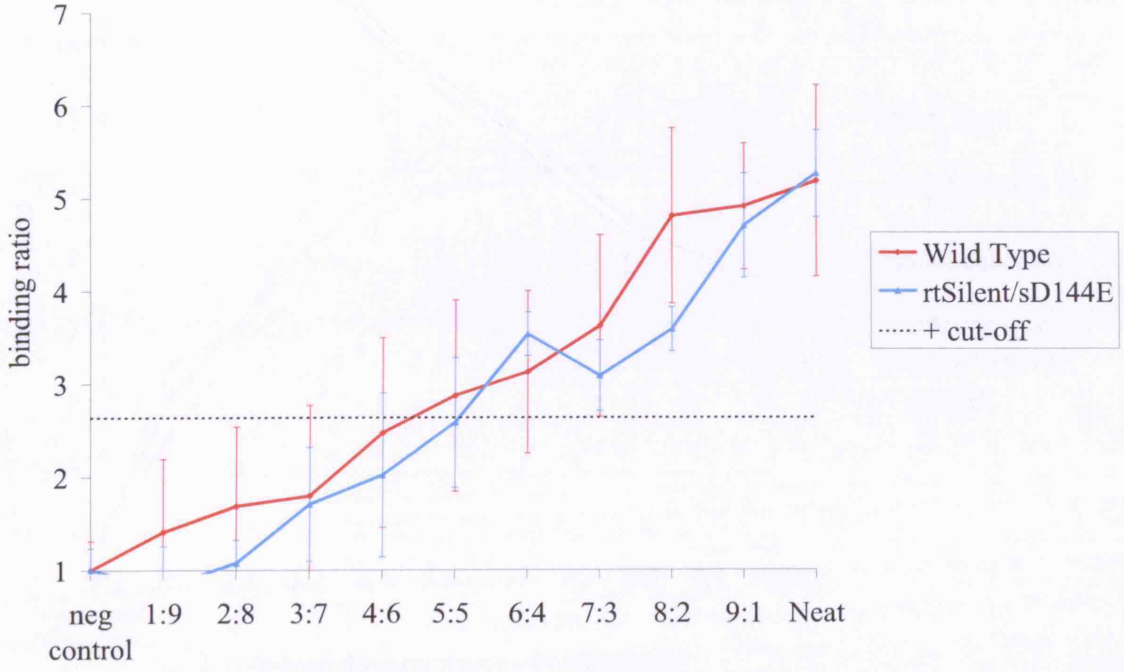


Figure A1.61 – Mean binding ratios of rtSilent/sD144E +rtM204V/sI195M mutant HBsAg in single monoclonal, multiple monoclonal and polyclonal capture ELISAs.

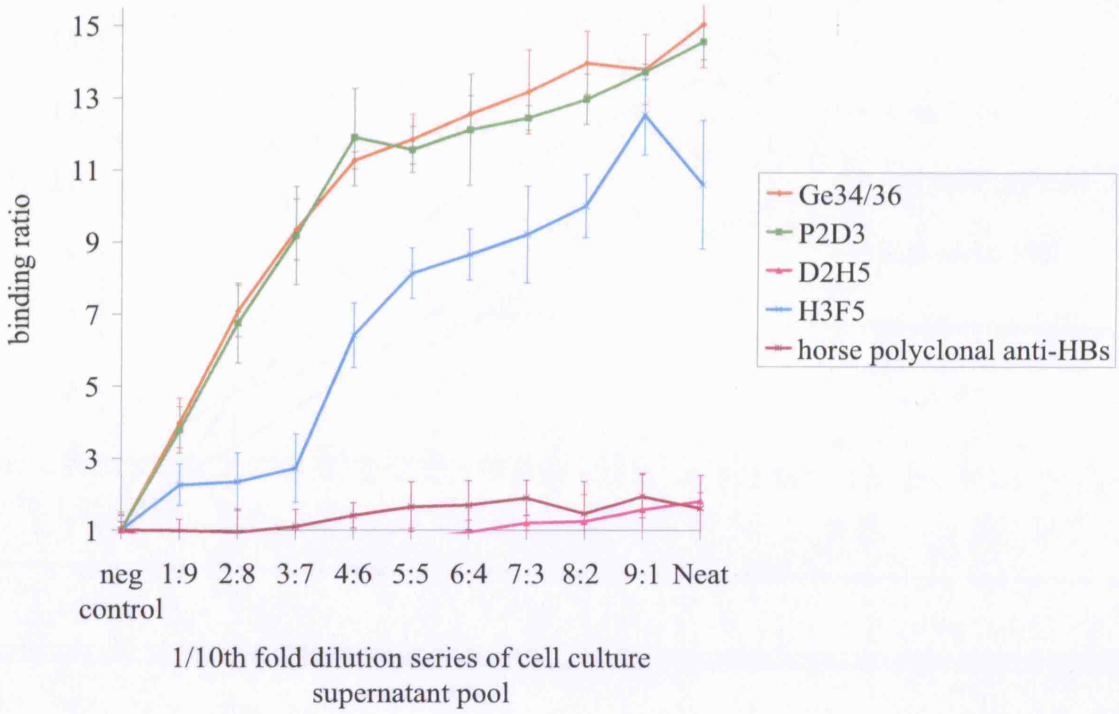


Figure A1.62 – Mean binding ratios of rtSilent/sD144E + rtM204V/sI195M mutant HBsAg in monoclonal P2D3 capture ELISA.

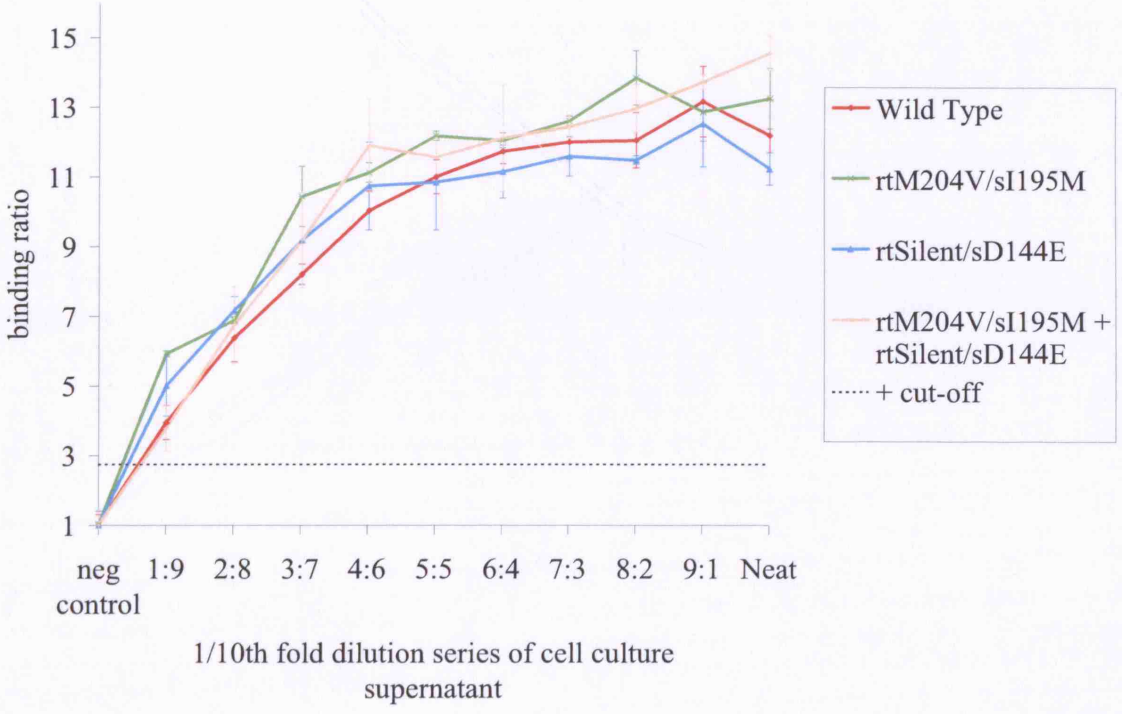


Figure A1.63 – Mean binding ratios of rtSilent/sD144E + rtM204V/sI195M mutant HBsAg in monoclonal D2H5 capture ELISA.

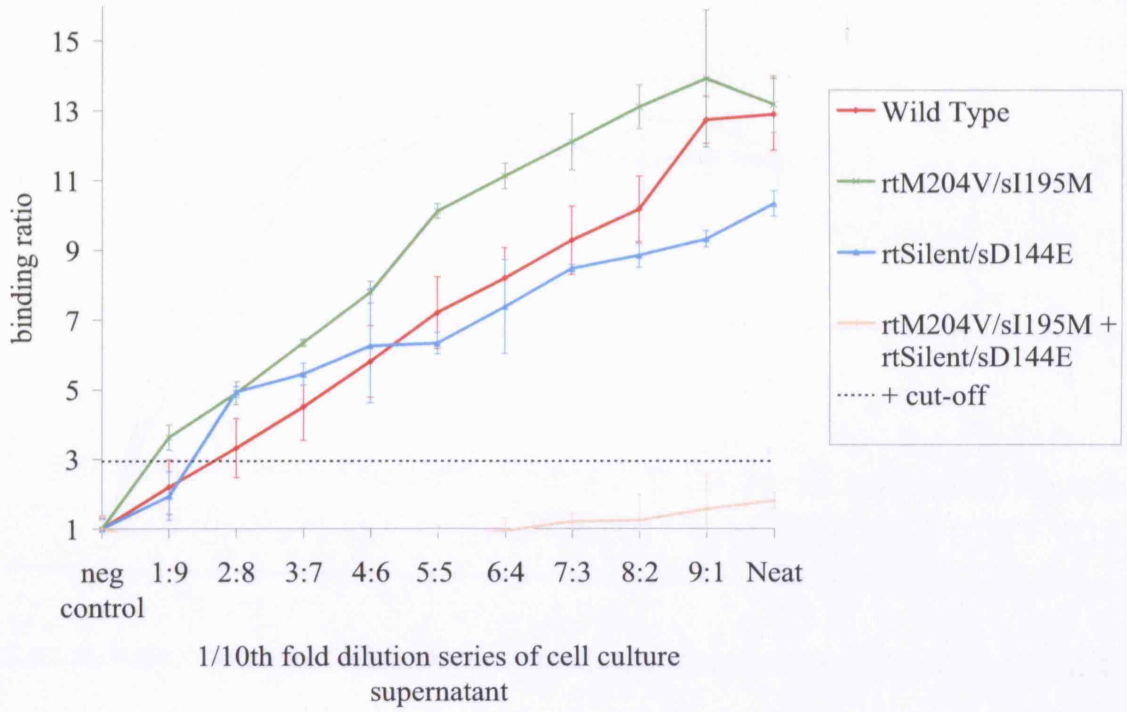


Figure A1.64 – Mean binding ratios of rtSilent/sD144E + rtM204V/sI195M mutant HBsAg in monoclonal H3F5 capture ELISA.

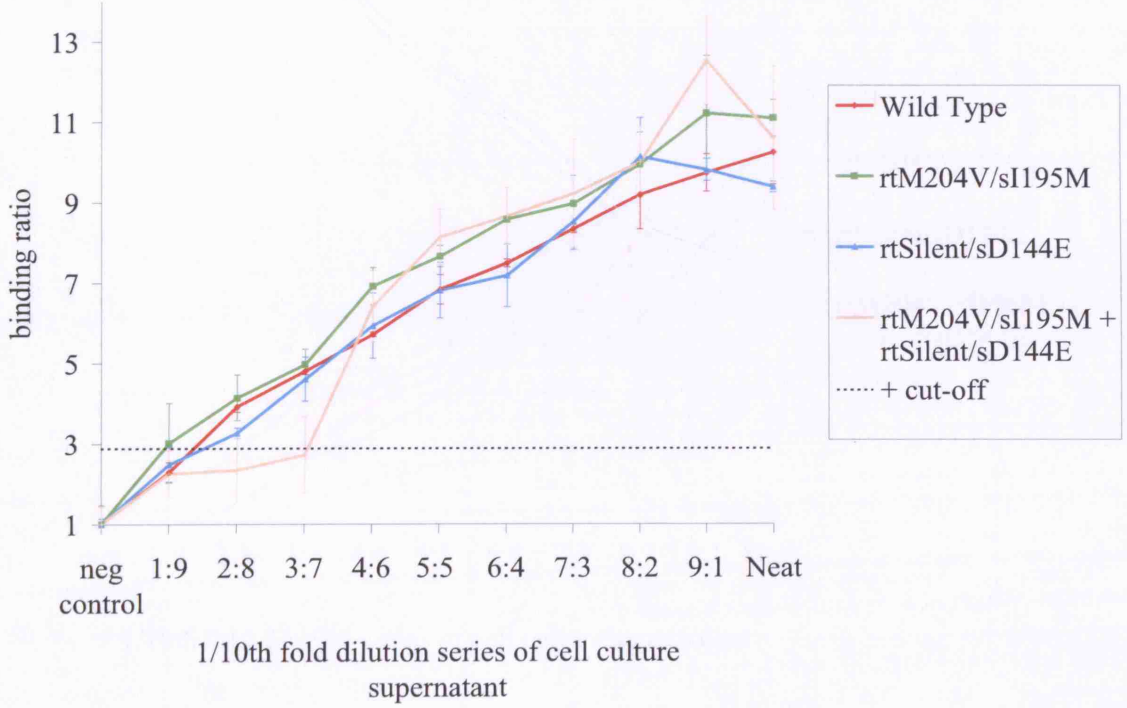


Figure A1.65 – Mean binding ratios of rtSilent/sD144E + rtM204V/sI195M mutant HBsAg in Ge34/36 format capture ELISA.

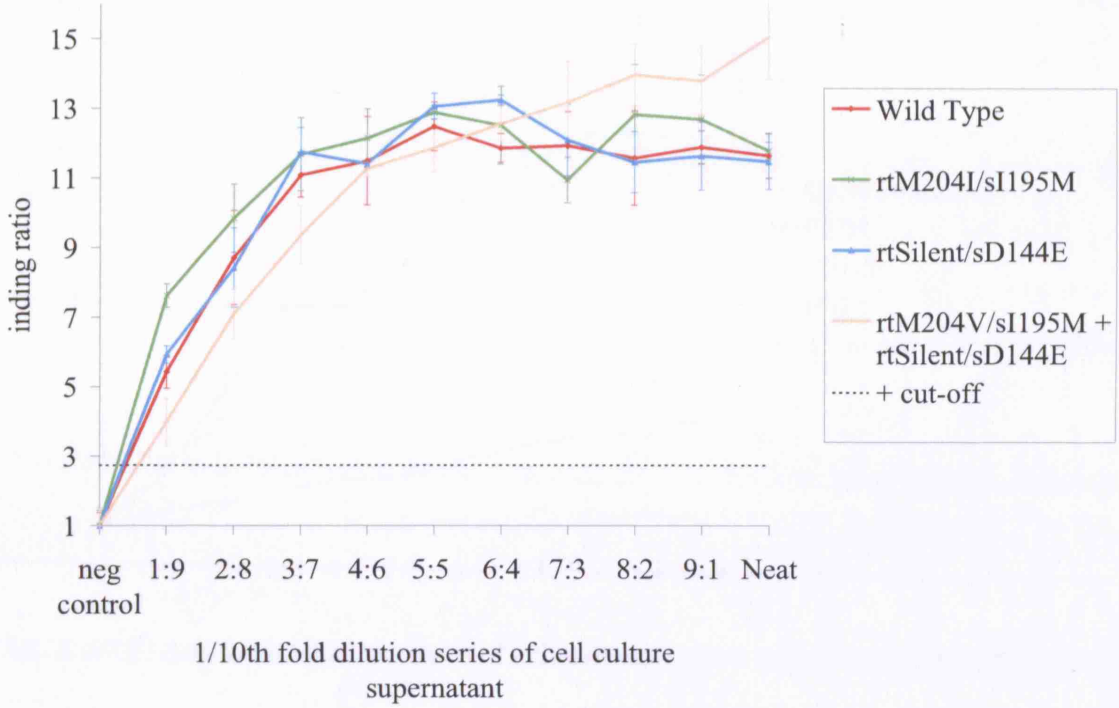


Figure A1.66– Mean binding ratios of rtSilent/sD144E + rtM204V/sI195M mutant HBsAg in horse polyclonal anti-HBs capture ELISA.

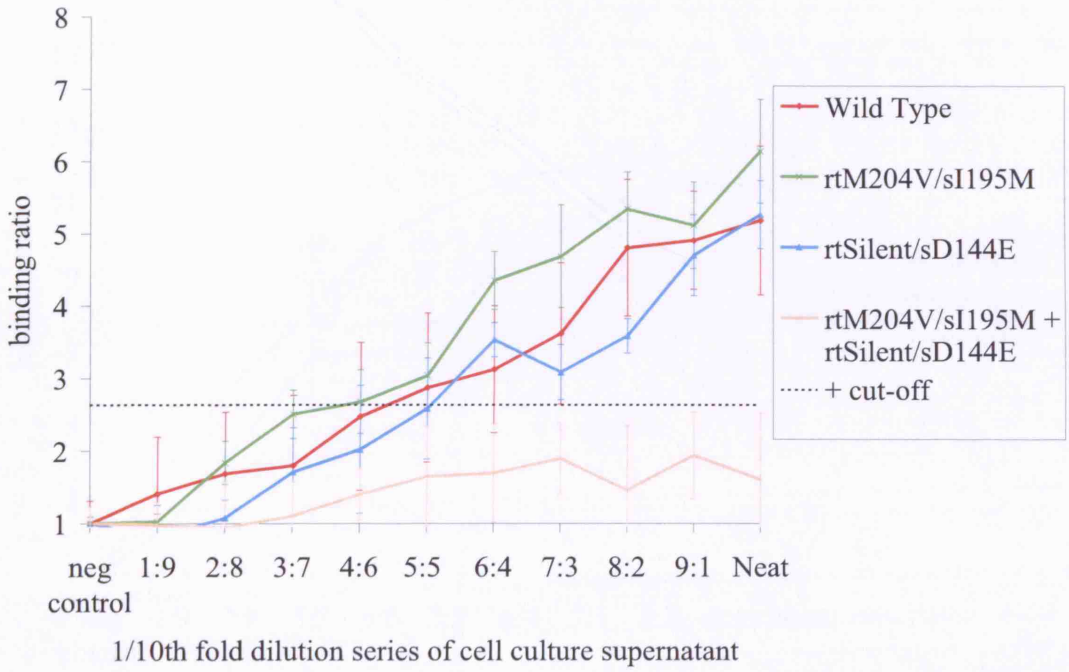


Figure A1.67 – Mean binding ratios of rtR153Q/sG145R mutant HBsAg in single monoclonal, multiple monoclonal and polyclonal capture ELISAs.

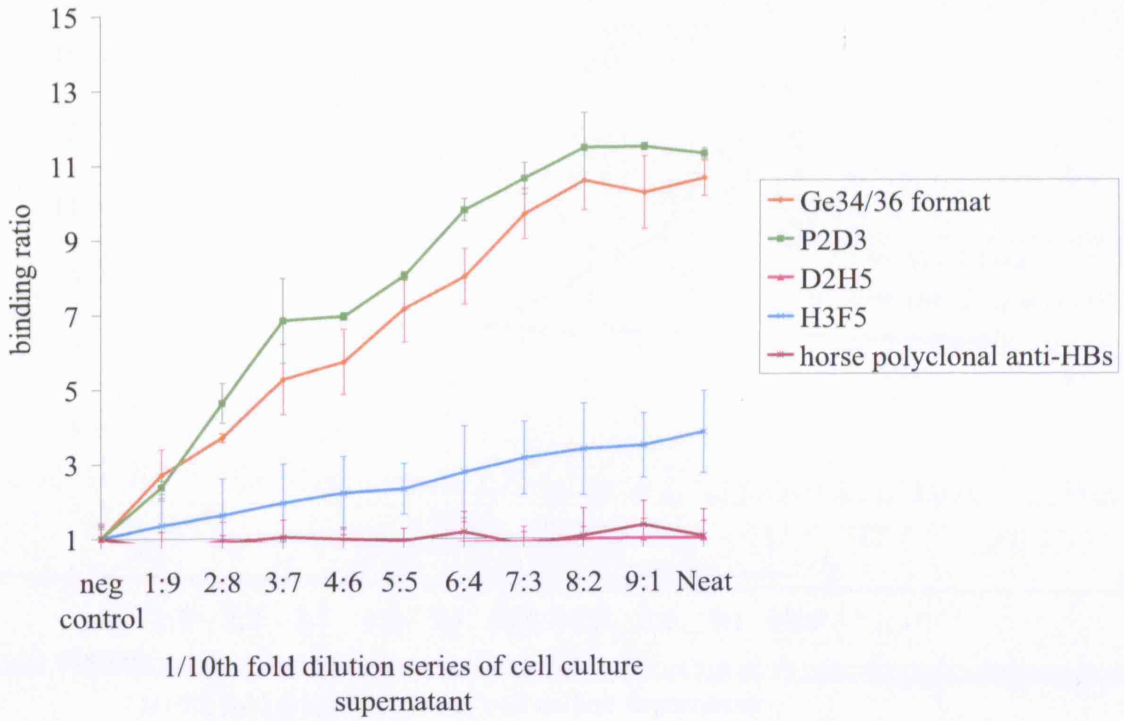


Figure A1.68 – Mean binding ratios of rtR153Q/sG145R mutant HBsAg in monoclonal P2D3 capture ELISA.

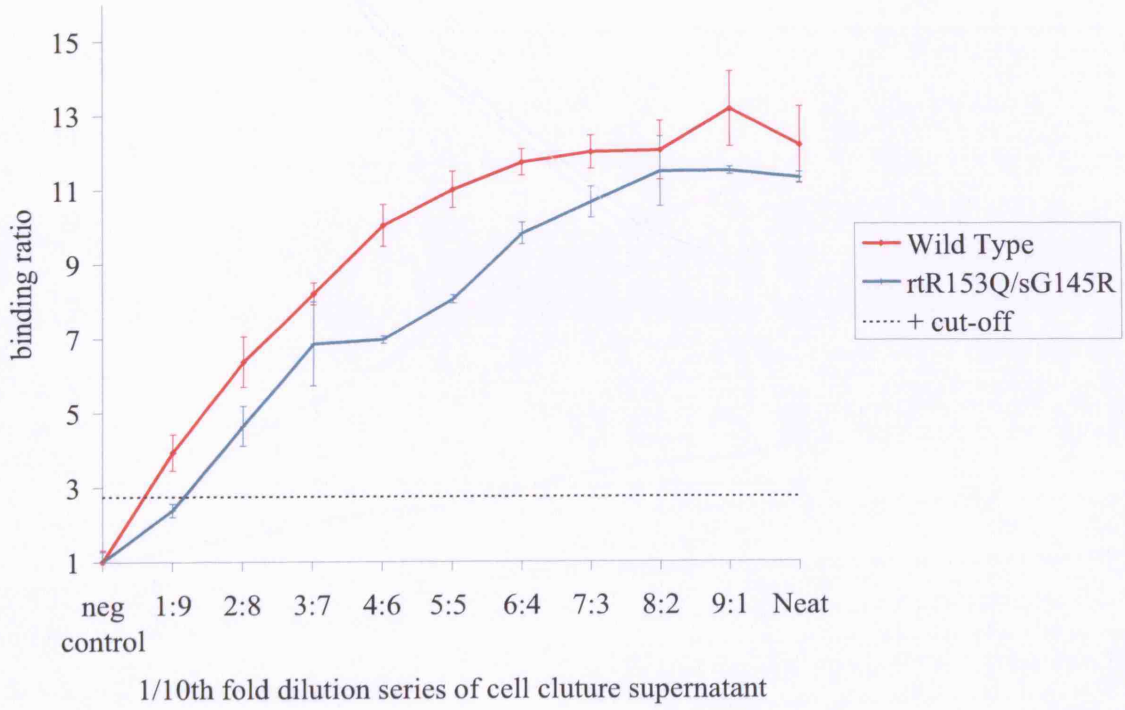


Figure A1.69 – Mean binding ratios of rtR153Q/sG145R mutant HBsAg in monoclonal D2H5 capture ELISA.

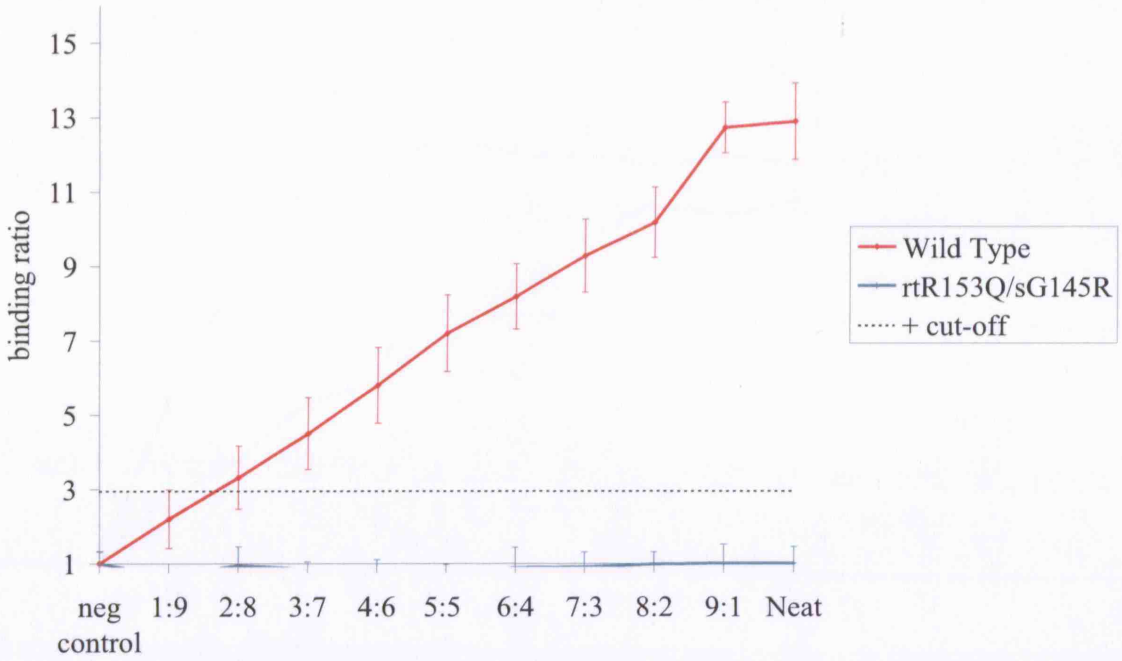


Figure A1.70 – Mean binding ratios of rtR153Q/sG145R mutant HBsAg in monoclonal H3F5 capture ELISA.

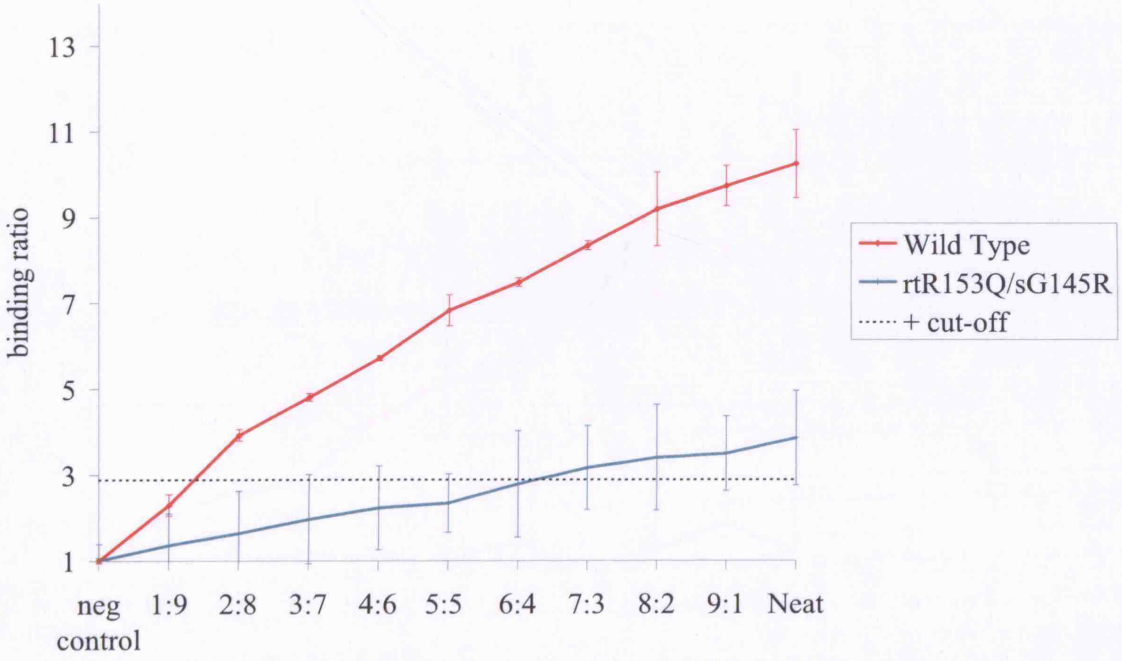


Figure A1.71 – Mean binding ratios of rtR153Q/sG145R mutant HBsAg in Ge34/36 format capture ELISA.

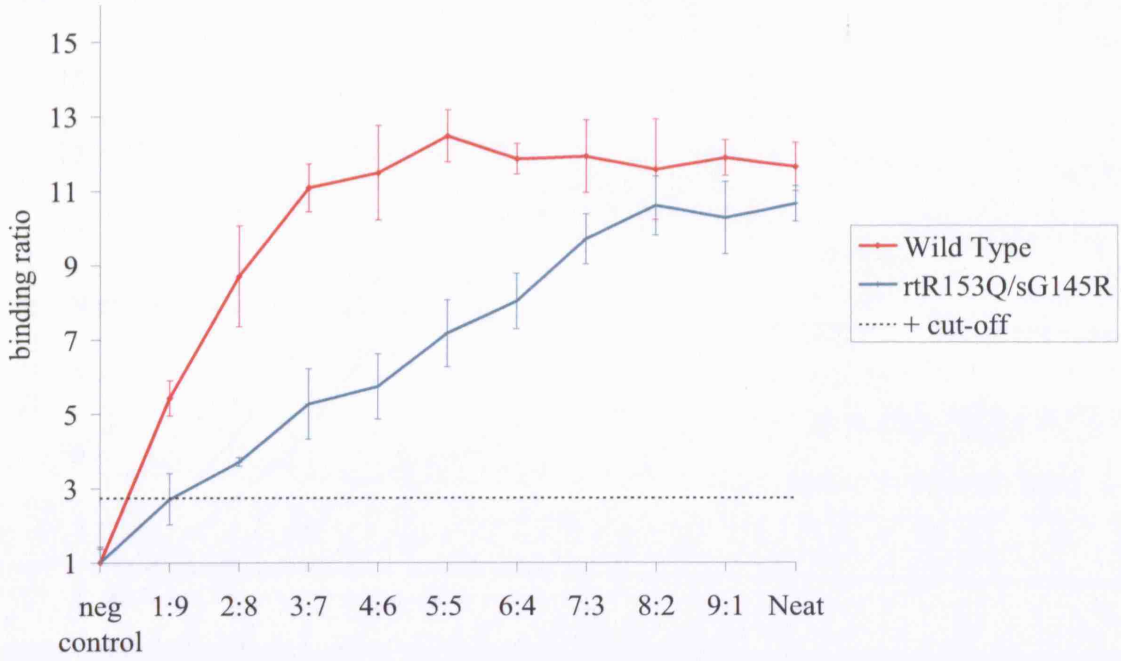


Figure A1.72– Mean binding ratios of rtR153Q/sG145R mutant HBsAg in horse polyclonal anti-HBs capture ELISA.

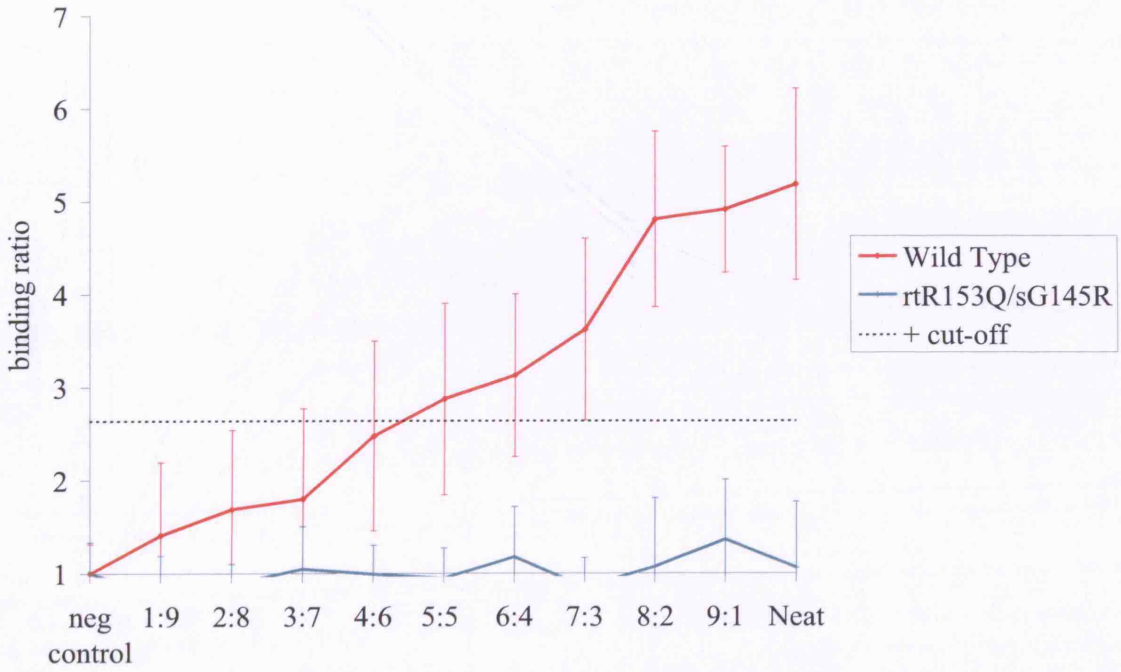


Figure A1.73 – Mean binding ratios of rtR153Q/sG145R +rtM204V/sI195M mutant HBsAg in single monoclonal, multiple monoclonal and polyclonal capture ELISAs.

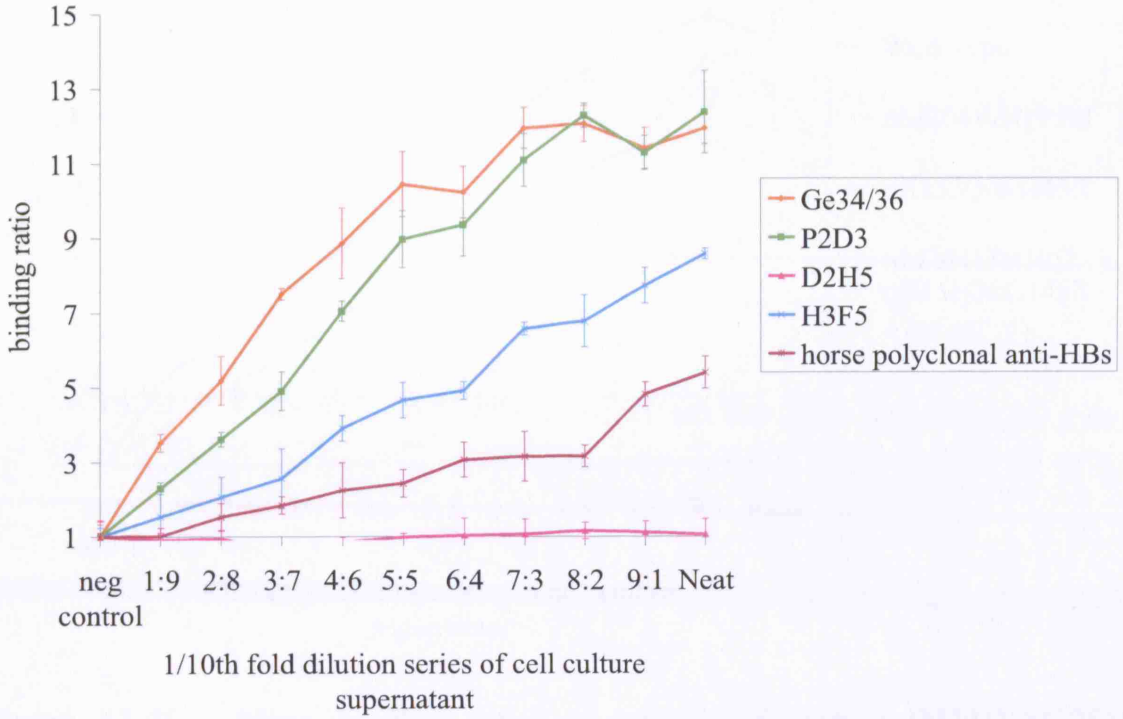


Figure A1.74 – Mean binding ratios of rtR153Q/sG145R +rtM204V/sI195M mutant HBsAg in monoclonal P2D3 capture ELISA.

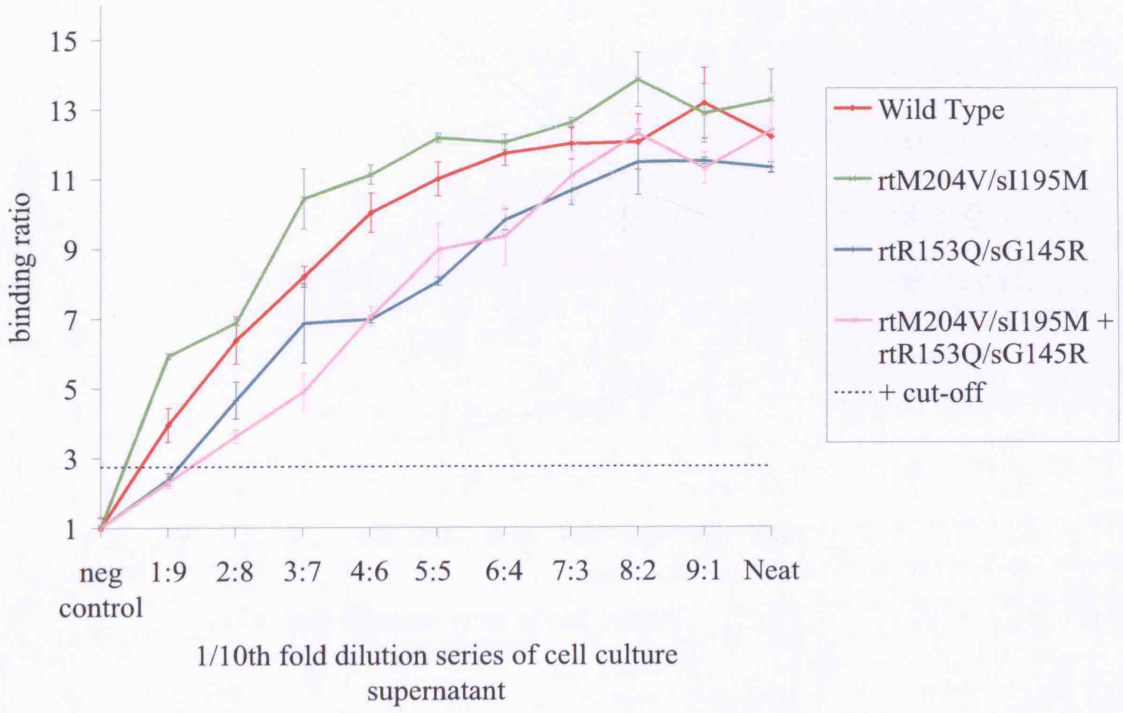


Figure A1.75 – Mean binding ratios of rtR153Q/sG145R +rtM204V/sI195M mutant HBsAg in monoclonal D2H5 capture ELISA.

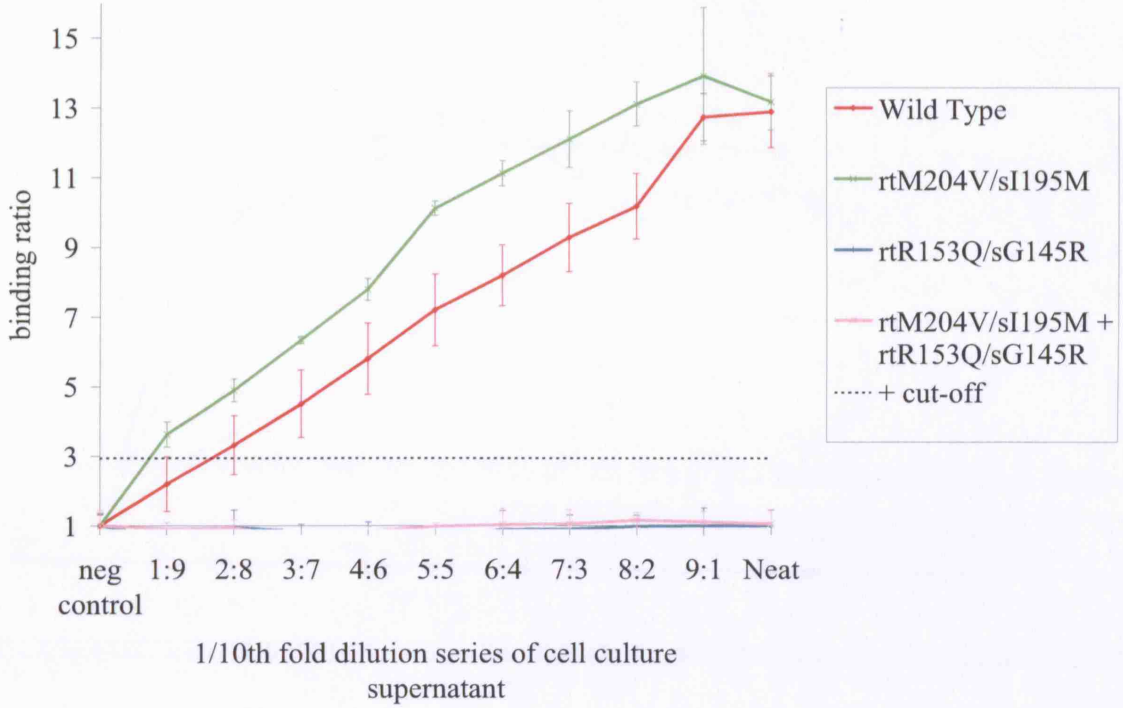


Figure A1.76 – Mean binding ratios of rtR153Q/sG145R +rtM204V/sI195M mutant HBsAg in monoclonal H3F5 capture ELISA.

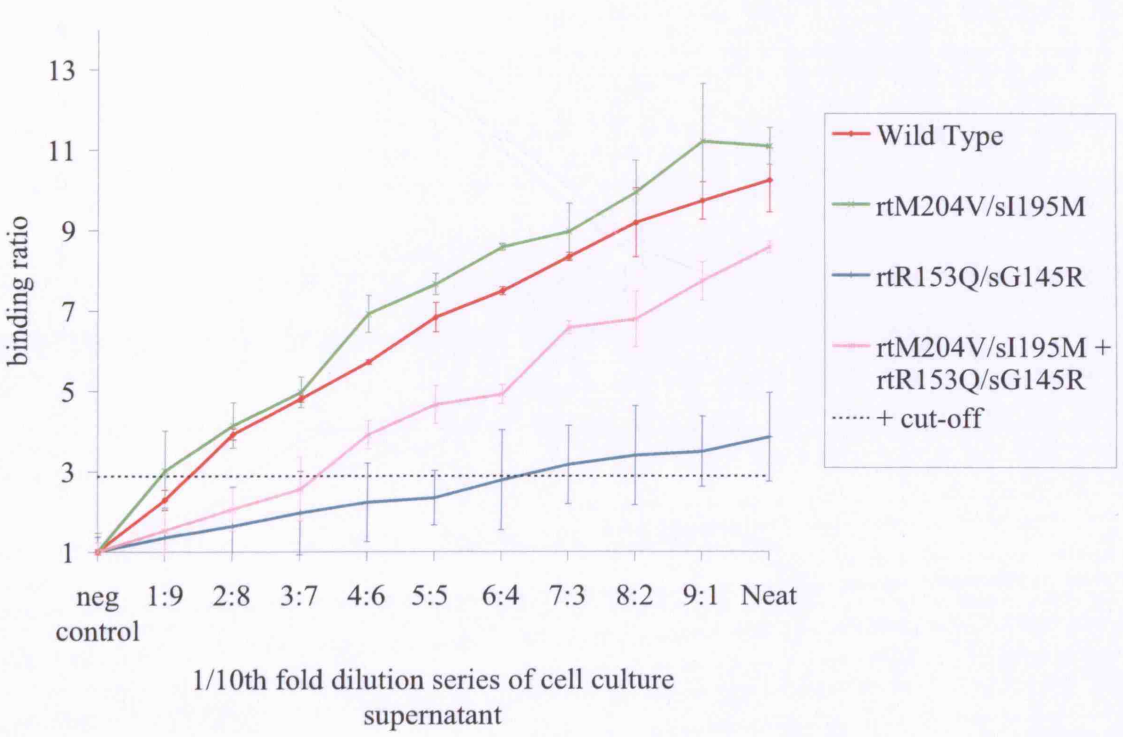


Figure A1.77 – Mean binding ratios of rtR153Q/sG145R +rtM204V/sI195M mutant HBsAg in Ge34/36 format capture ELISA.

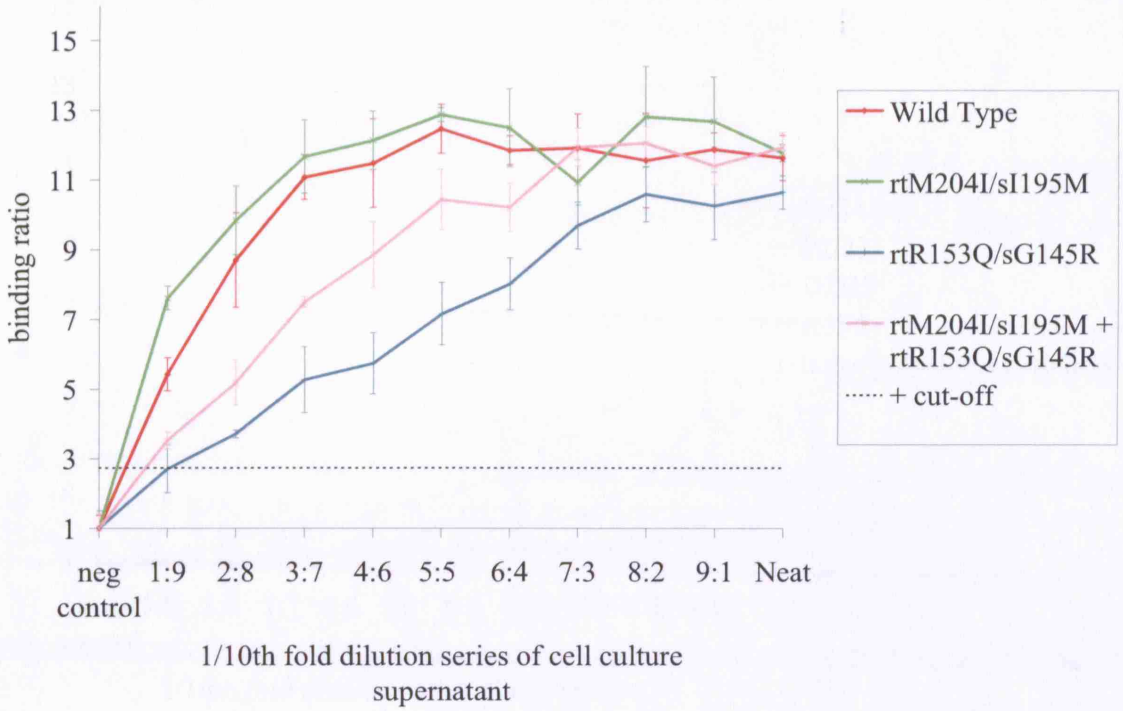


Figure A1.78– Mean binding ratios of rtR153Q/sG145R +rtM204V/sI195M mutant HBsAg in horse polyclonal anti-HBs capture ELISA.

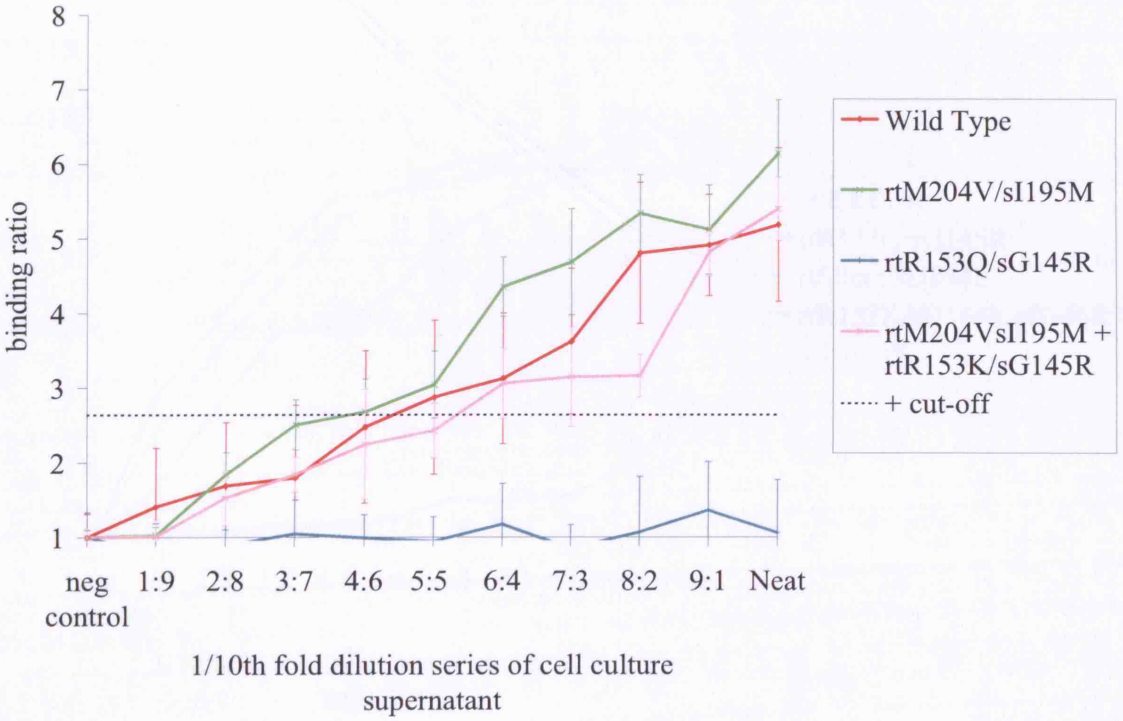


Figure A1.79 – Mean binding ratios of rtR153K/sD144E+sG145R mutant HBsAg in single monoclonal, multiple monoclonal and polyclonal capture ELISAs.

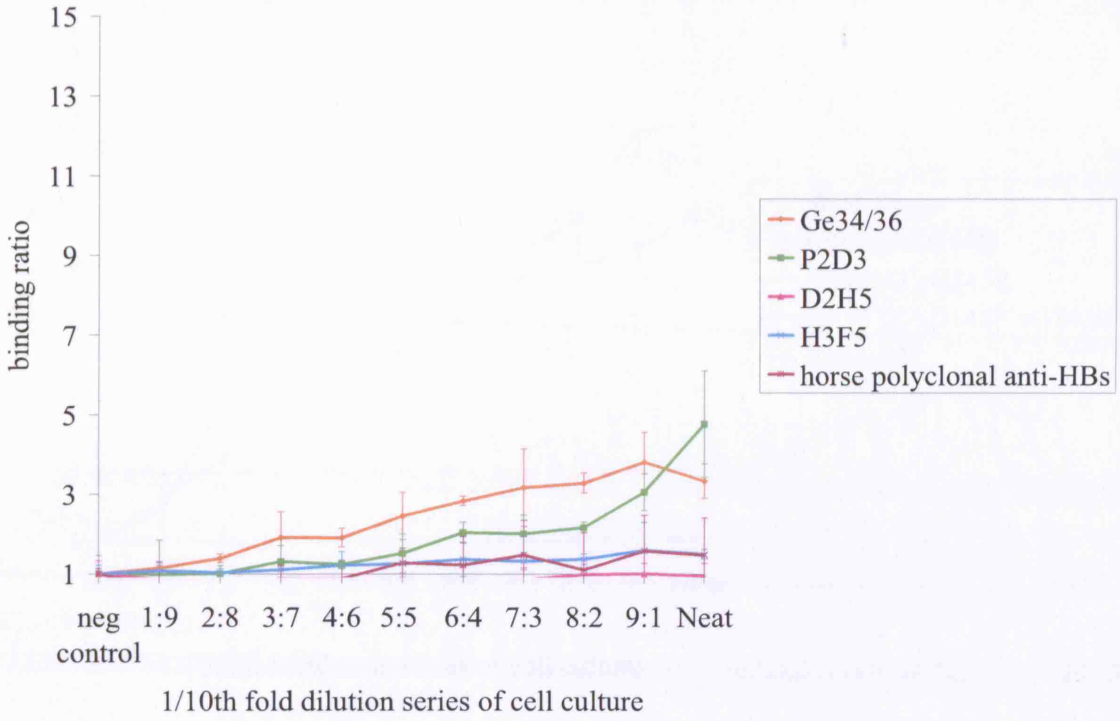


Figure A1.80 – Mean binding ratios of rtR153K/sD144E+sG145R mutant HBsAg in monoclonal P2D3 capture ELISA.

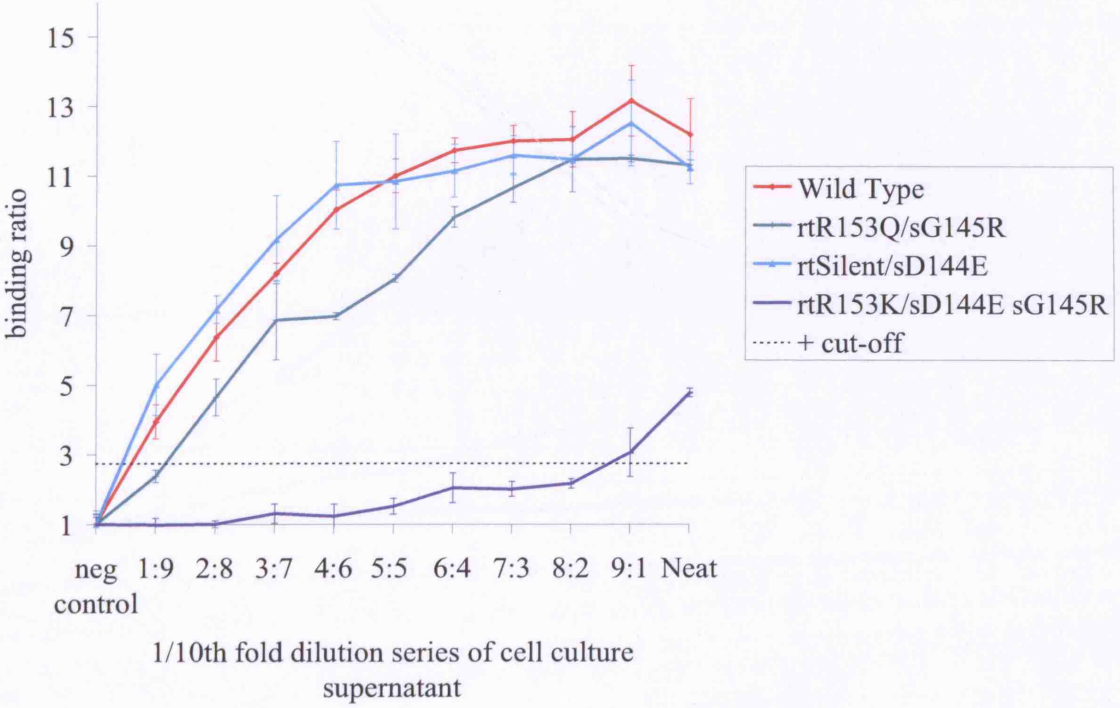


Figure A1.81 – Mean binding ratios of rtR153K/sD144E+sG145R mutant HBsAg in monoclonal D2H5 capture ELISA.

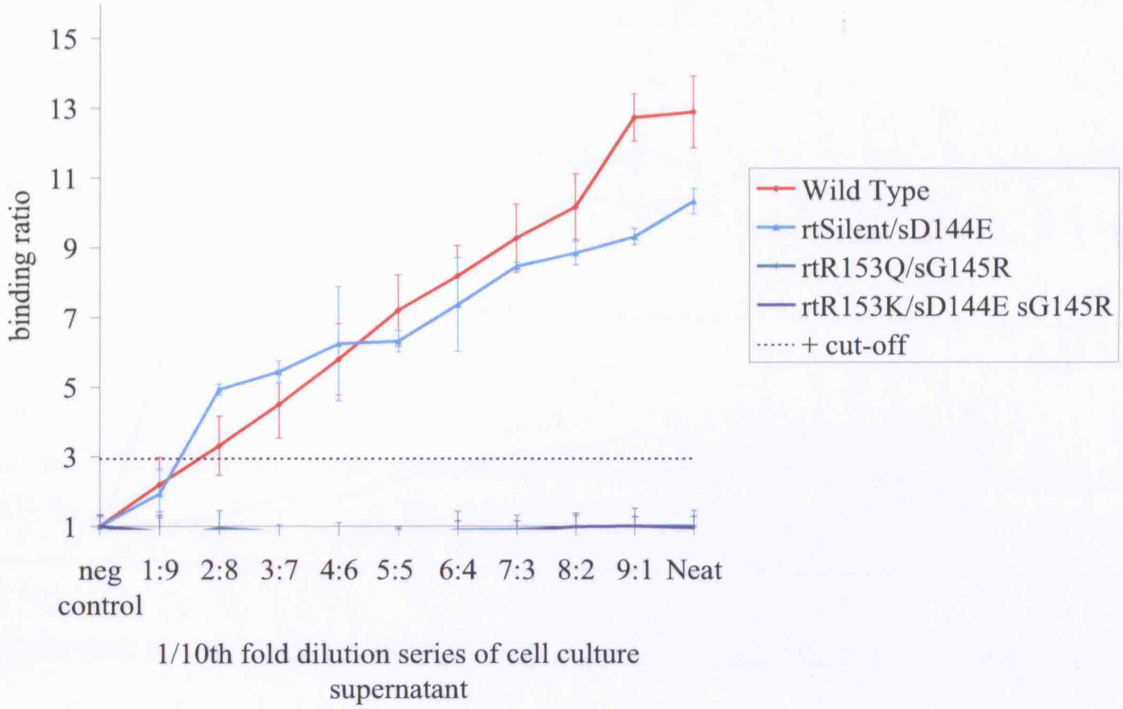


Figure A1.82 – Mean binding ratios of rtR153K/sD144E+sG145R mutant HBsAg in monoclonal H3F5 capture ELISA.

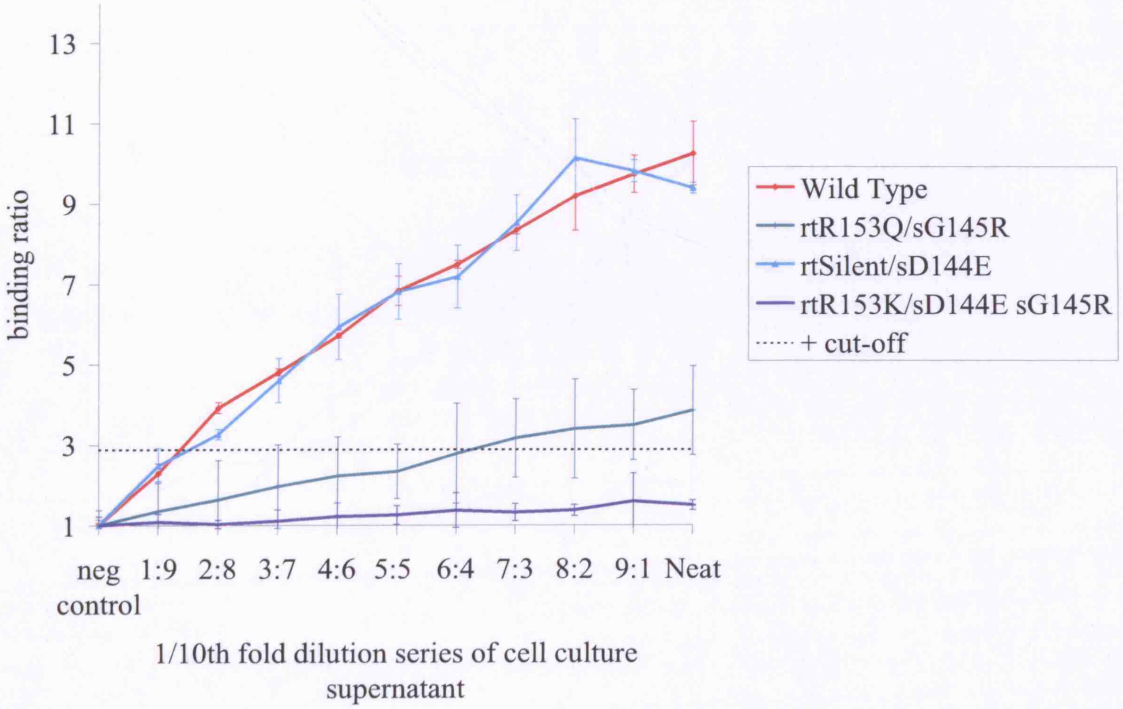


Figure A1.83 – Mean binding ratios of rtR153K/sD144E+sG145R mutant HBsAg in Ge34/36 format capture ELISA.

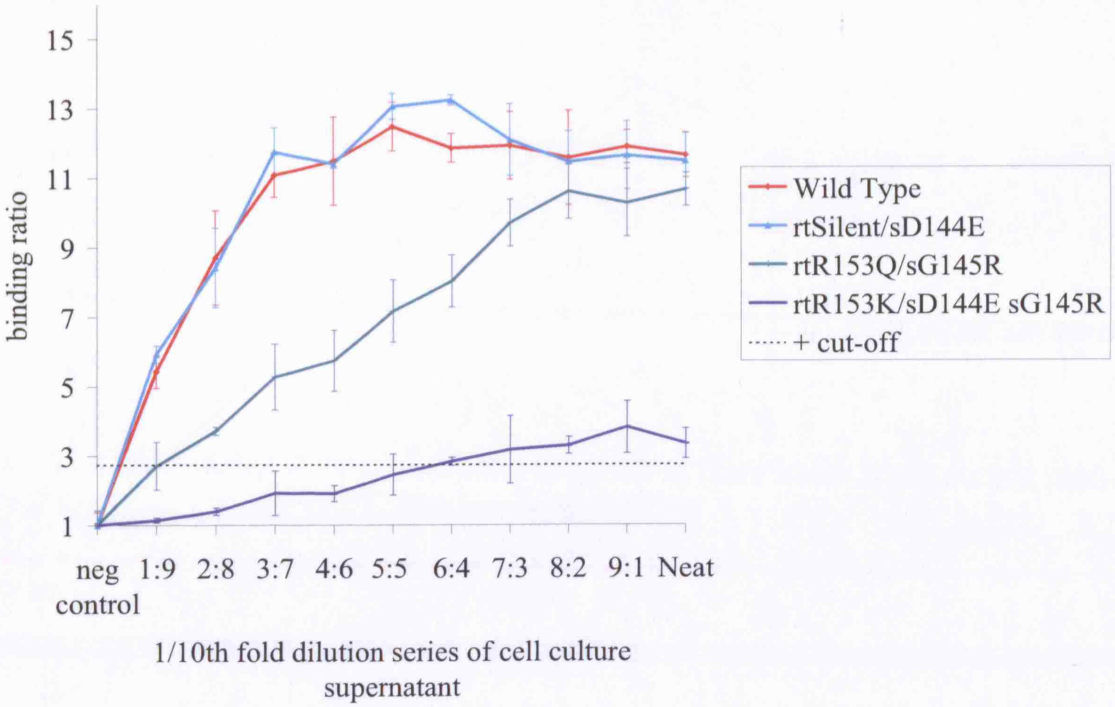


Figure A1.84– Mean binding ratios of rtR153K/sD144E+sG145R mutant HBsAg in horse polyclonal anti-HBs capture ELISA.

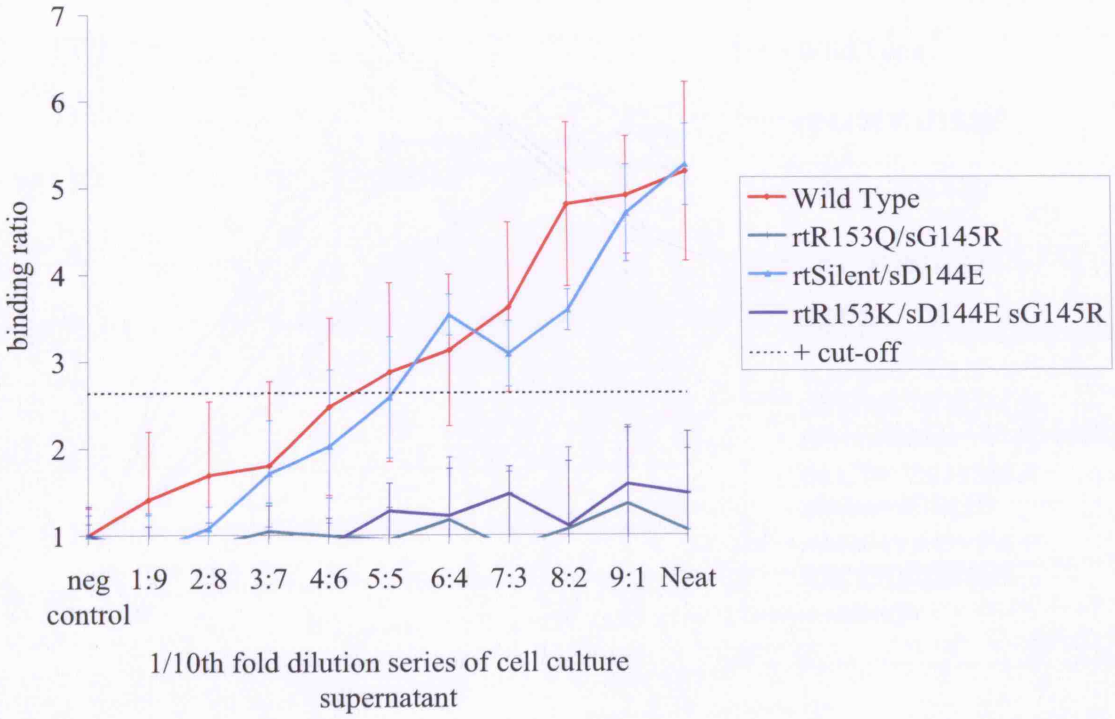


Figure A1.85 – Mean binding ratios of rtR153K/sD144E+sG145R + rtM204V/sI195M mutant HBsAg in all capture ELISAs.

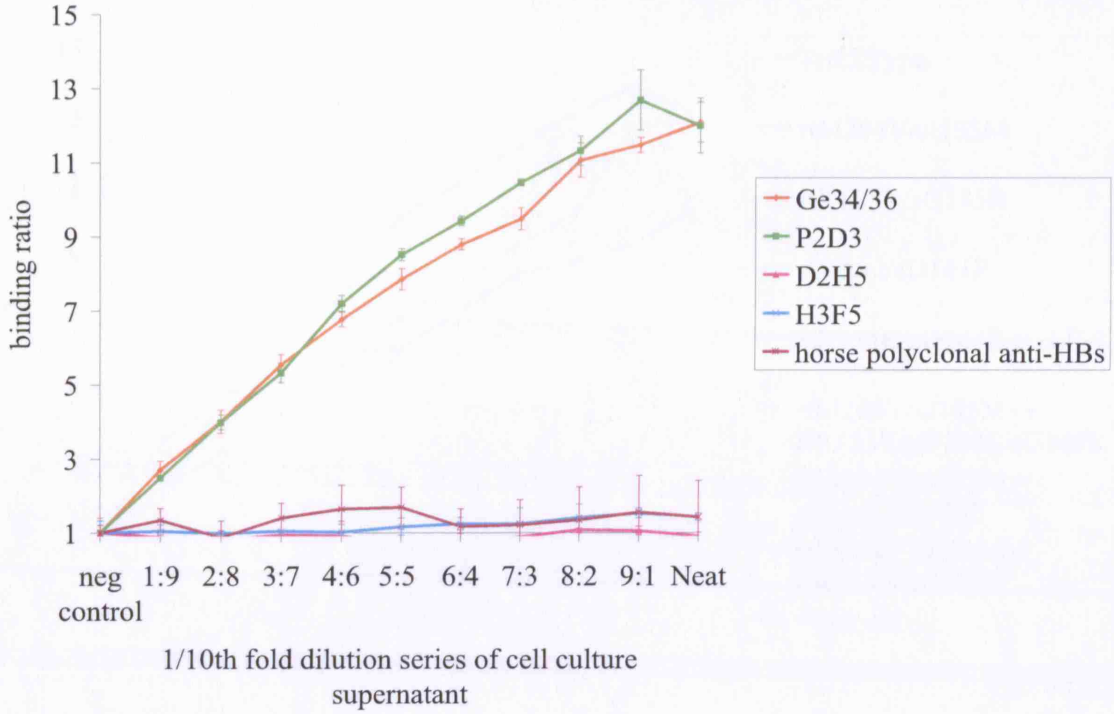


Figure A1.86 – Mean binding ratios of rtR153K/sD144E+sG145R + rtM204VsI195M mutant HBsAg in monoclonal P2D3 capture ELISA.

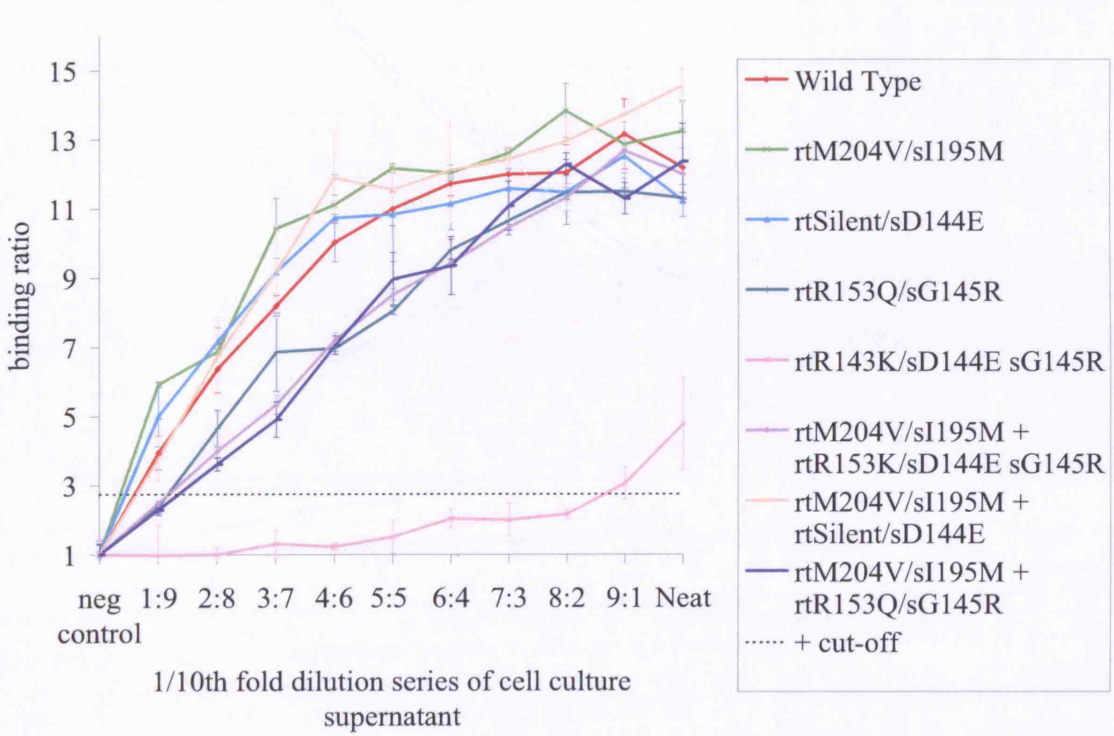


Figure A1.87 – Mean binding ratios of rtR153K/sD144E+sG145R + rtM204VsI195M mutant HBsAg in monoclonal D2H5 capture ELISA.

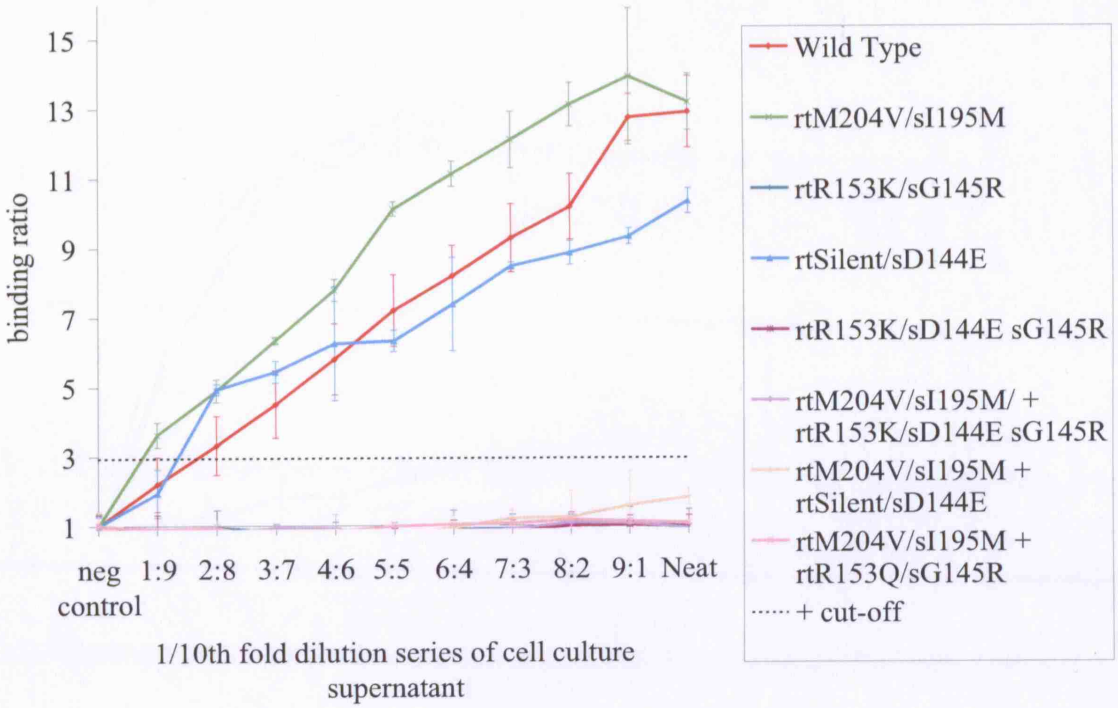


Figure A1.88 – Mean binding ratios of rtR153K/sD144E+sG145R + rtM204VsI195M mutant HBsAg in monoclonal H3F5 capture ELISA.

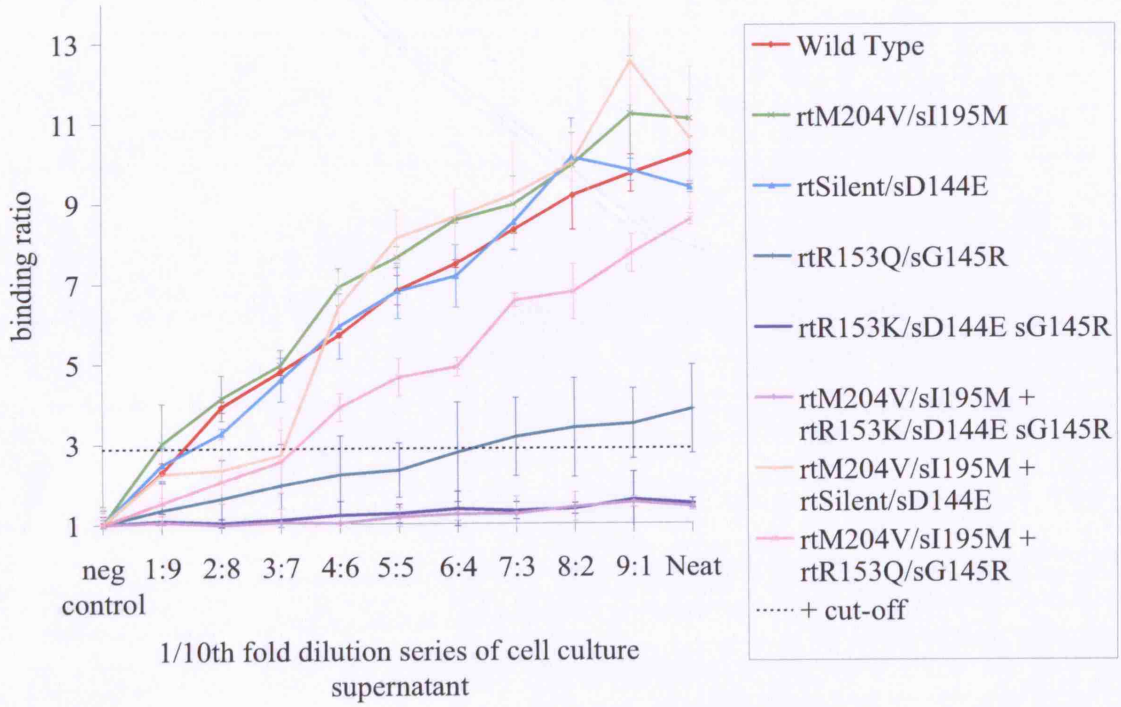


Figure A1.89 – Mean binding ratios of rtR153K/sD144E+sG145R + rtM204VsI195M mutant HBsAg in Ge34/36 format capture ELISA.

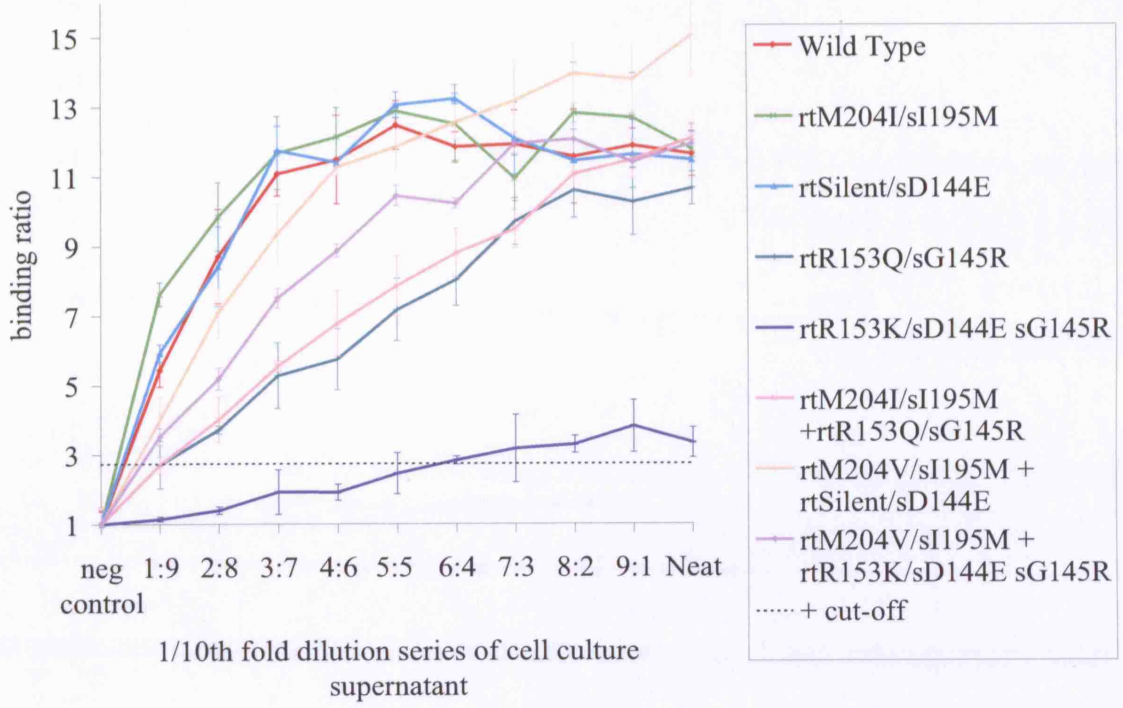


Figure A1.90– Mean binding ratios of rtR153K/sD144E+sG145R + rtM204VsI195M mutant HBsAg in horse polyclonal anti-HBs capture ELISA.

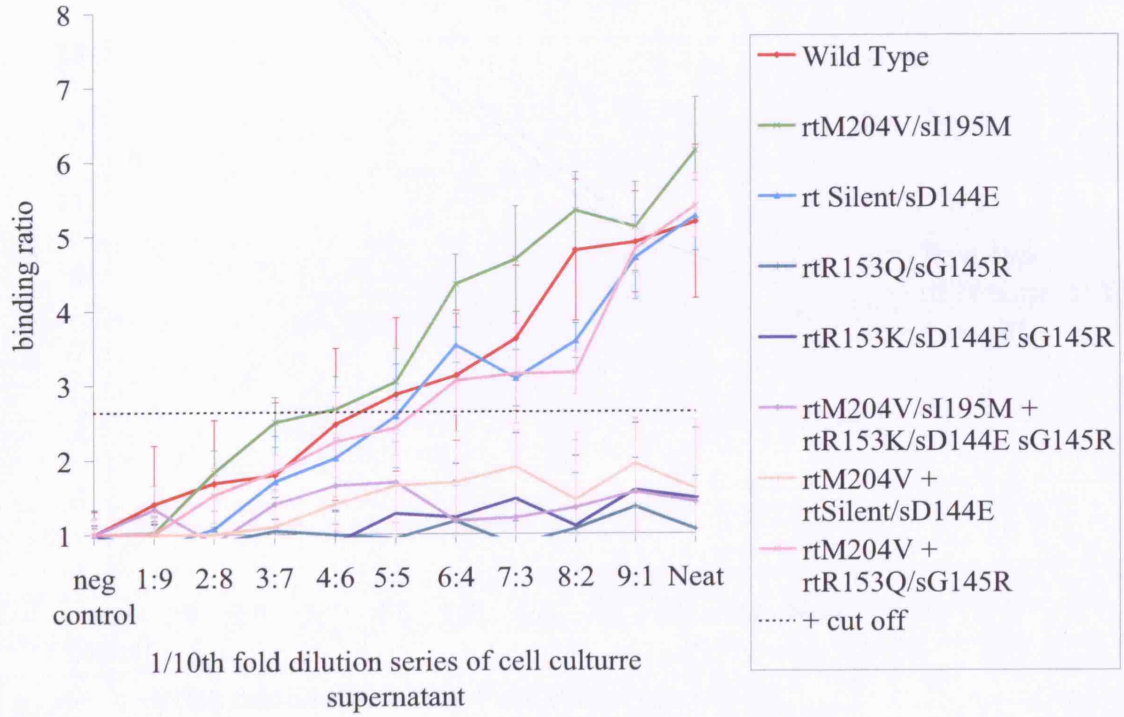


Figure A1.91 – Mean binding ratios of rtF166L/sF158Y mutant HBsAg in single monoclonal, multiple monoclonal and polyclonal capture ELISAs.

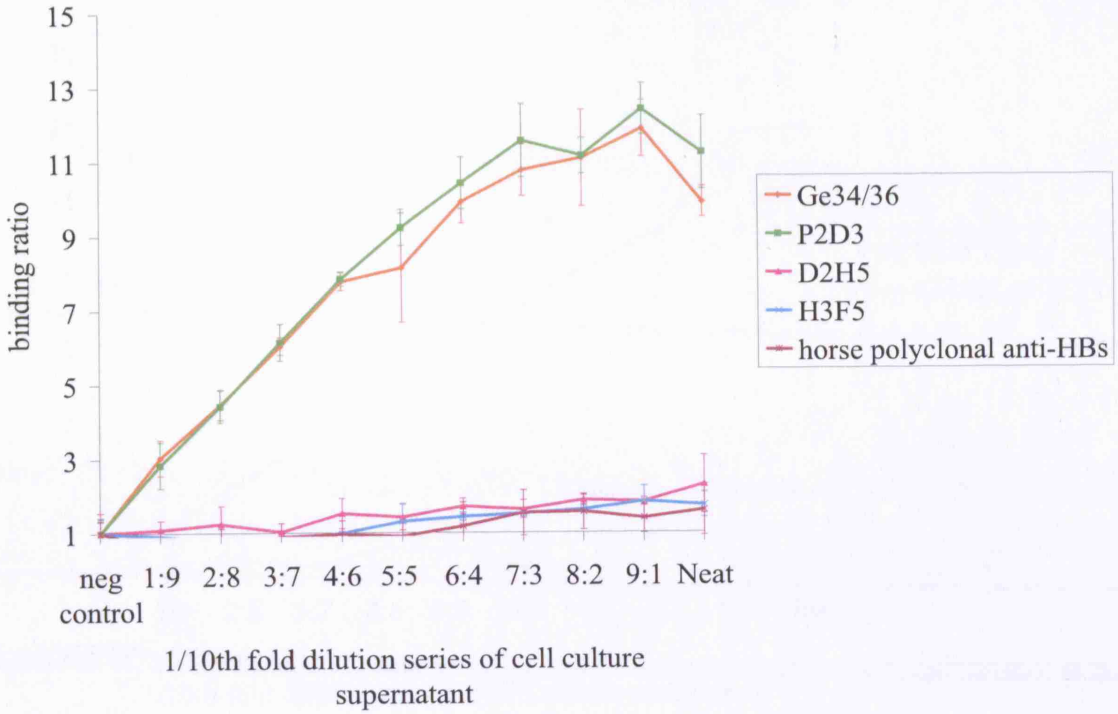


Figure A1.92 – Mean binding ratios of rtF166L/sF158Y mutant HBsAg in monoclonal P2D3 capture ELISA.

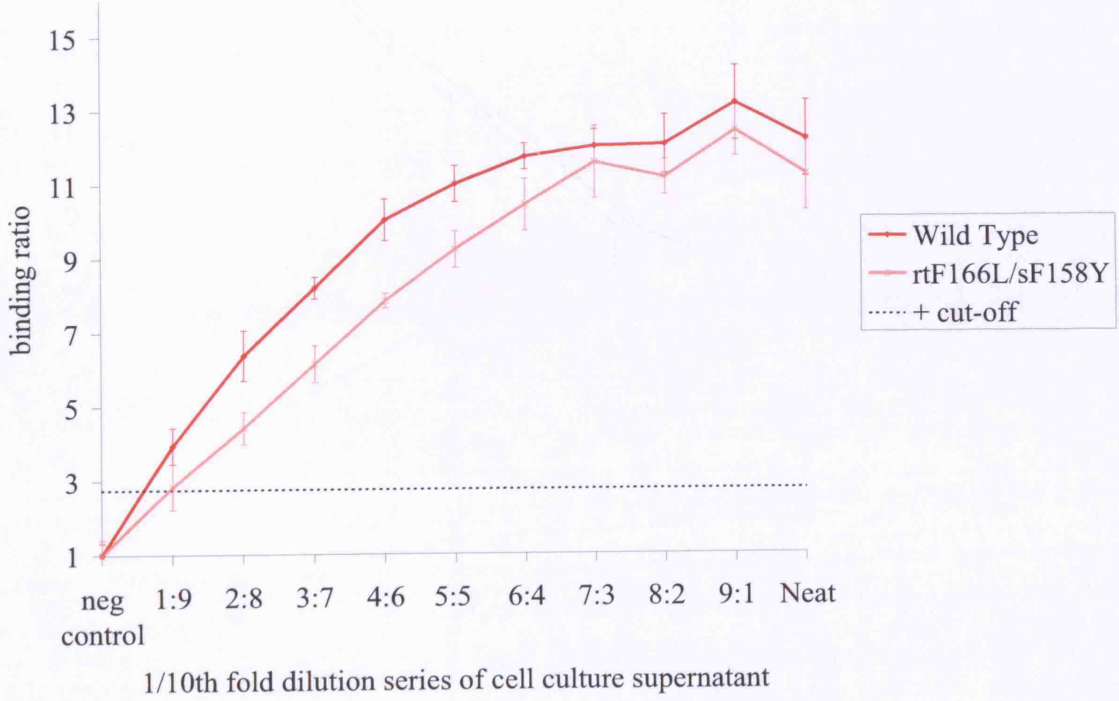


Figure A1.93 – Mean binding ratios of rtF166L/sF158Y mutant HBsAg in monoclonal D2H5 capture ELISA.

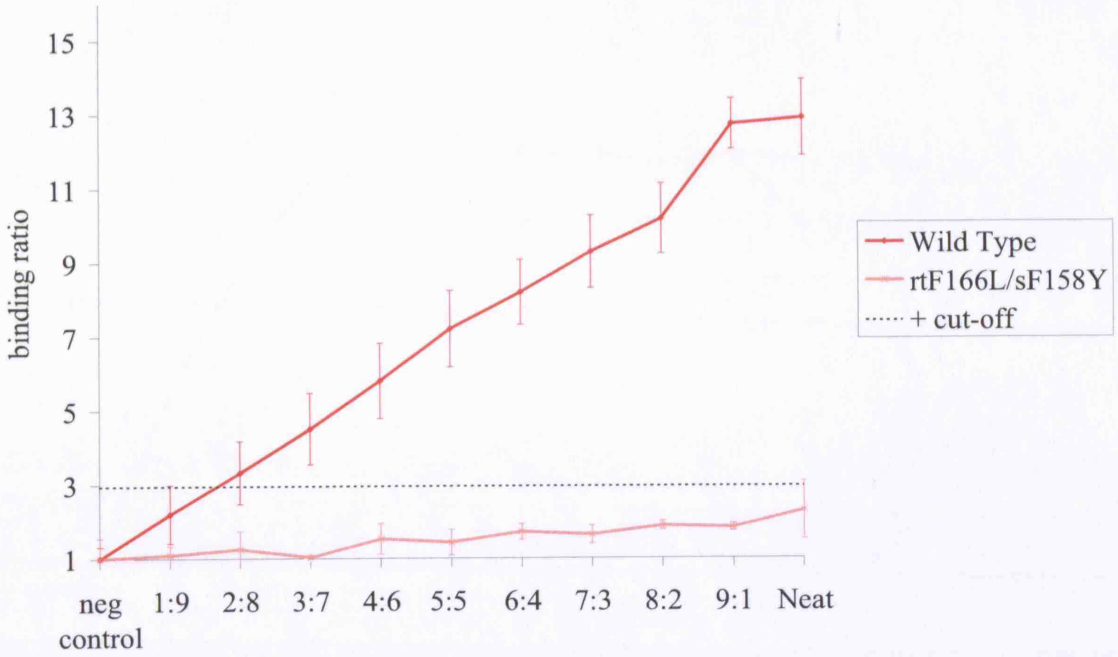


Figure A1.94 – Mean binding ratios of rtF166L/sF158Y mutant HBsAg in monoclonal H3F5 capture ELISA.

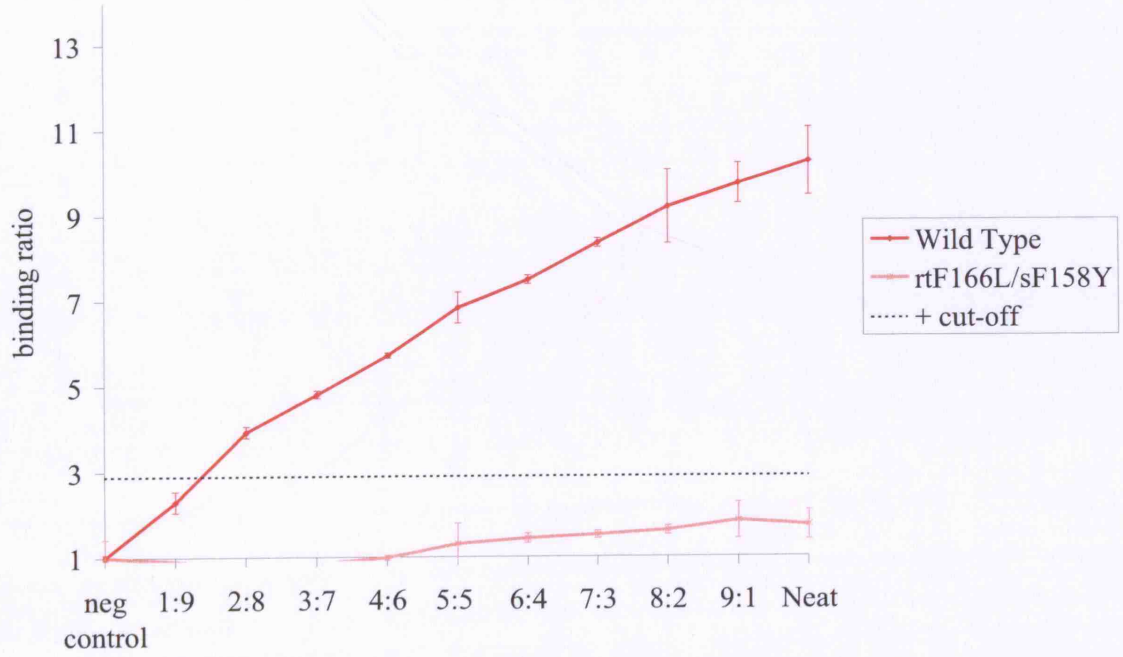


Figure A1.95 – Mean binding ratios of rtF166L/sF158Y mutant HBsAg in Ge34/36 format capture ELISA.

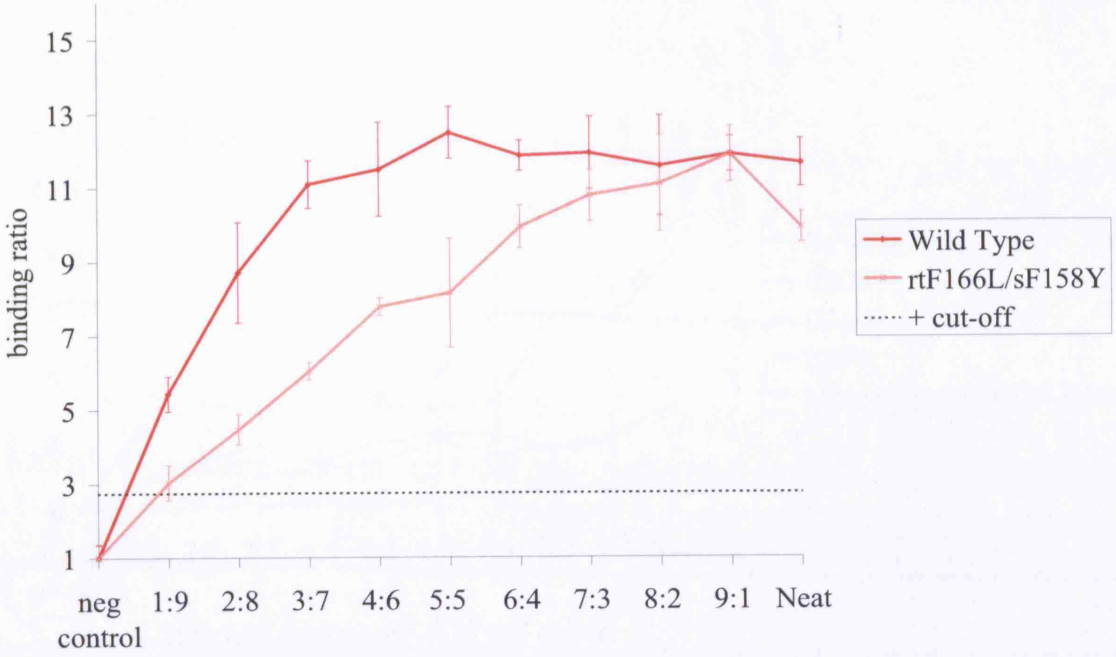


Figure A1.96– Mean binding ratios of rtF166L/sF158Y mutant HBsAg in horse polyclonal anti-HBs capture ELISA.

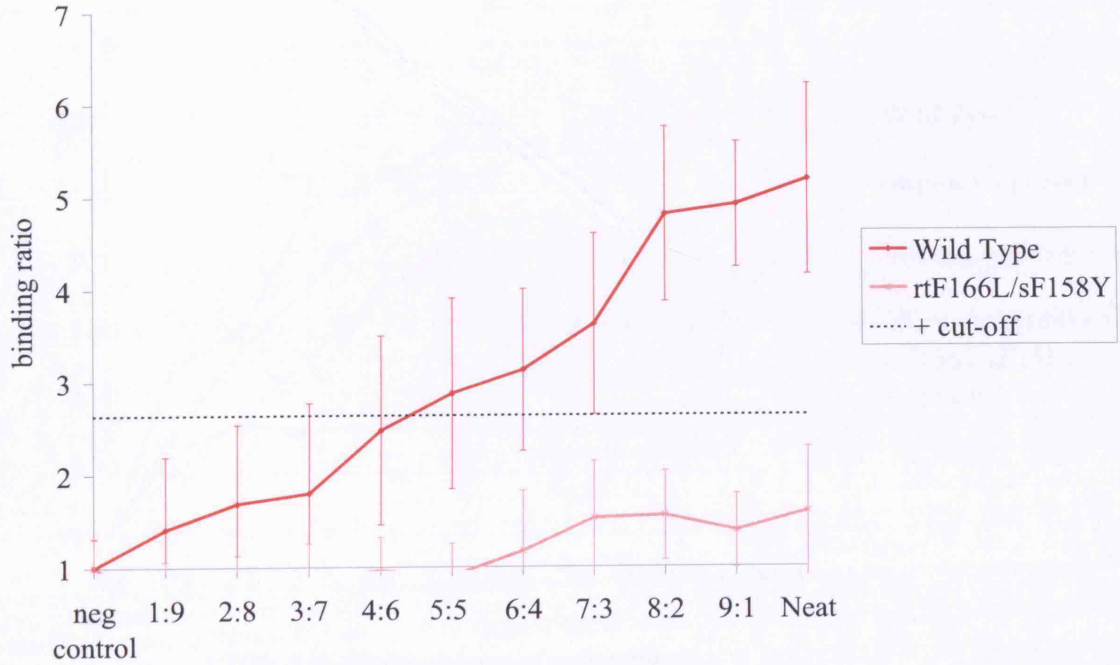


Figure A1.97 – Mean binding ratios of rtF166L/sF158Y +rtM204V/sI195M mutant HBsAg in single monoclonal, multiple monoclonal and polyclonal capture ELISAs.

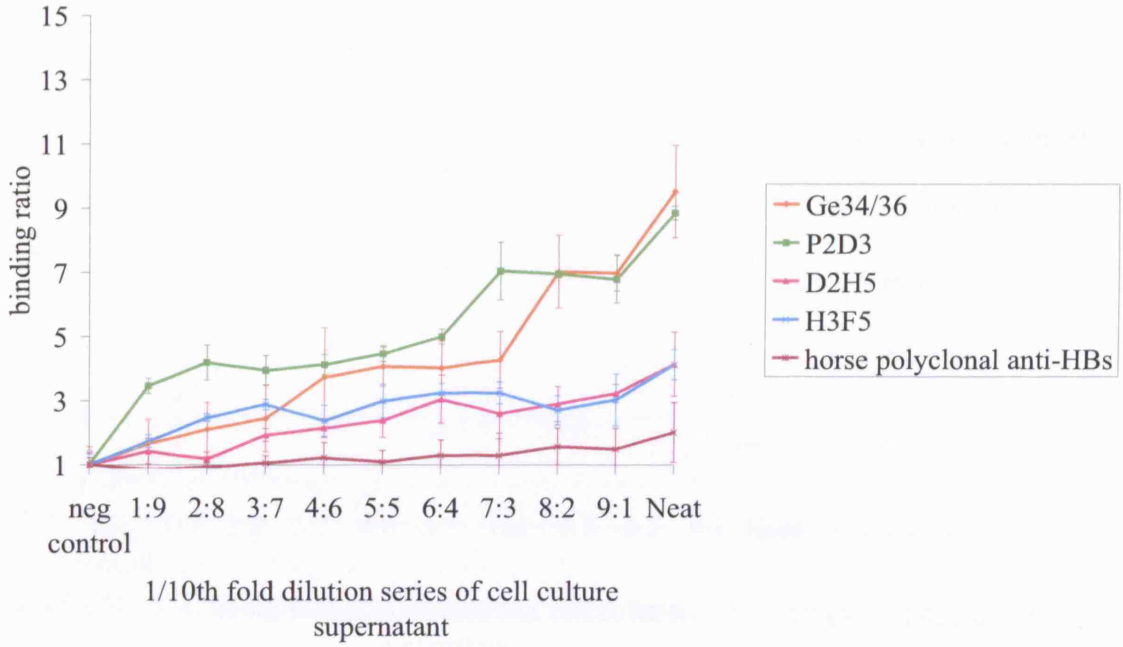


Figure A1.98 – Mean binding ratios of rtF166L/sF158Y +rtM204V/sI195M mutant HBsAg in monoclonal P2D3 capture ELISA.

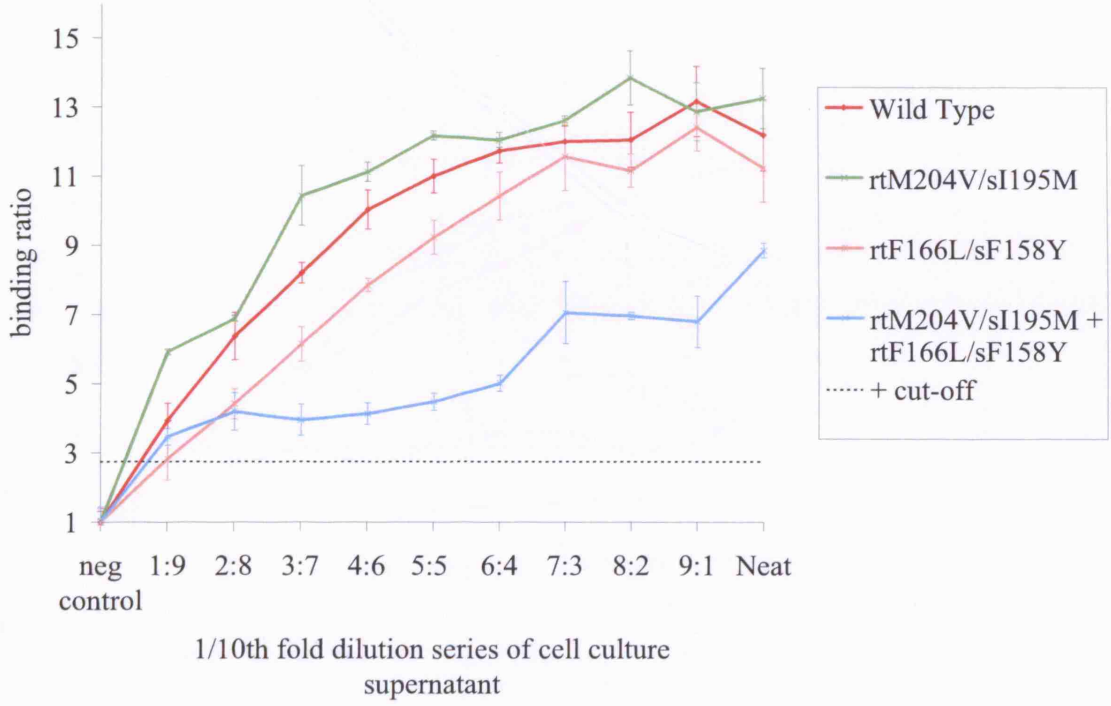


Figure A1.99 – Mean binding ratios of rtF166L/sF158Y +rtM204V/sI195M mutant HBsAg in monoclonal D2H5 capture ELISA.

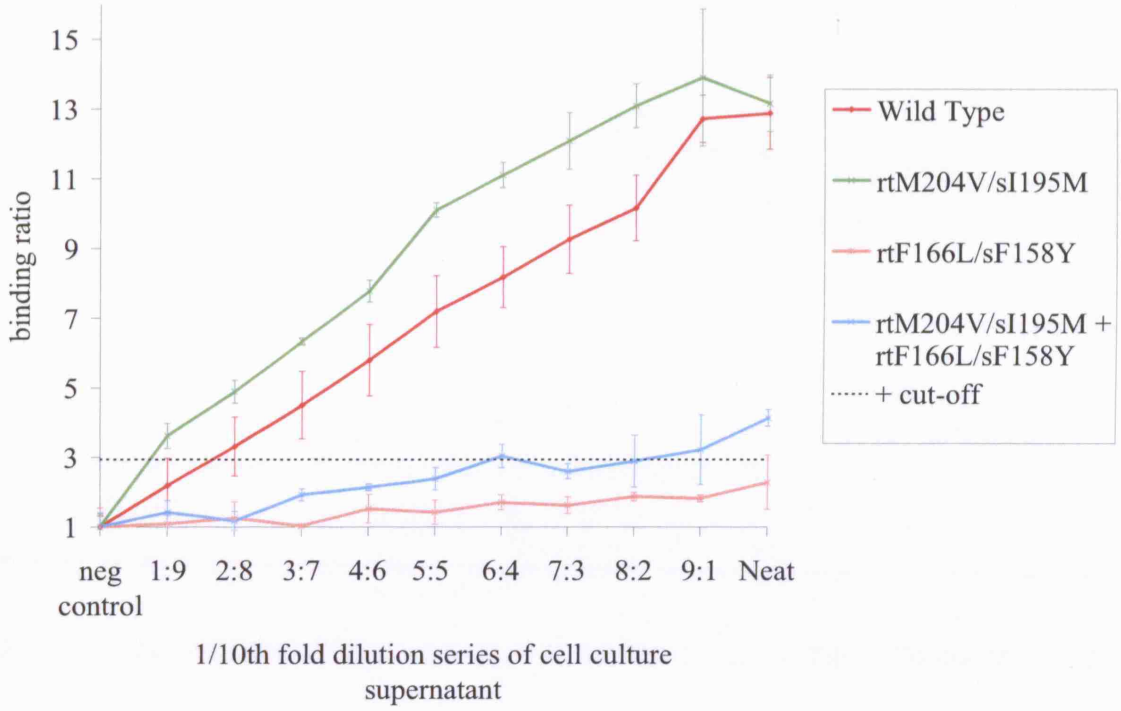
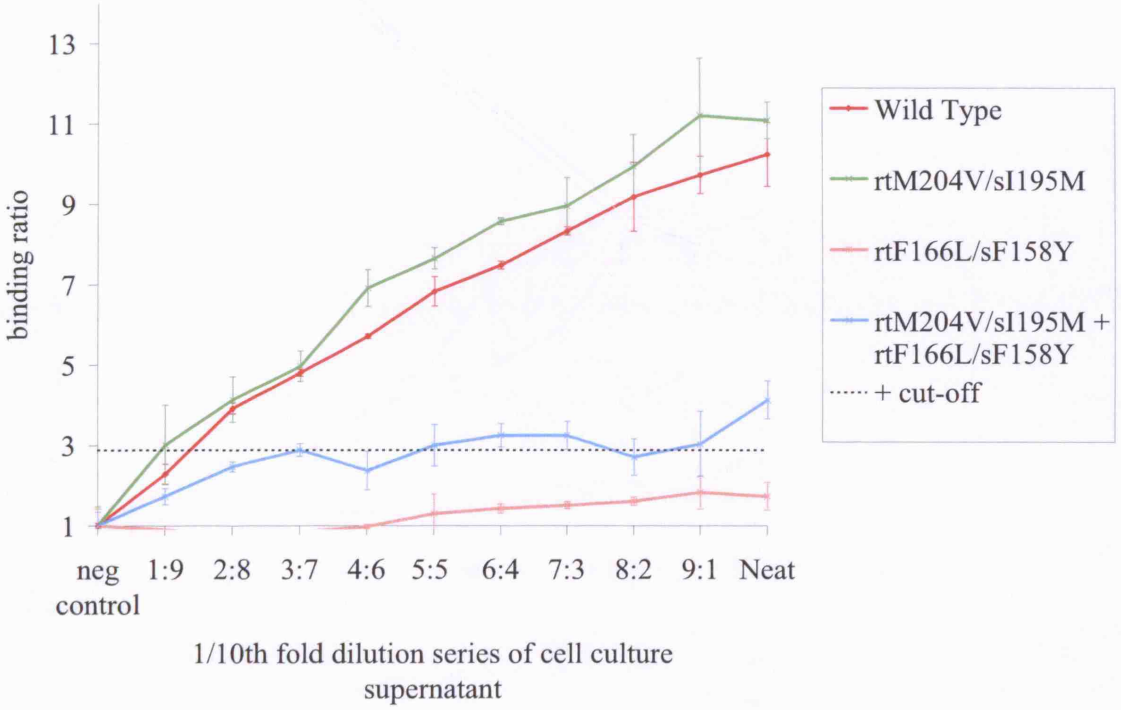


Figure A1.100 – Mean binding ratios of rtF166L/sF158Y +rtM204V/sI195M mutant HBsAg in monoclonal H3F5 capture ELISA.



APPENDIX A2

NUCLEOTIDE AND PREDICTED AMINO ACID SEQUENCES OF S GENES CLONED INTO pBK-CMV[smallS] CONSTRUCTS

A2.2 (overleaf) – Predicted aa sequence (in the S ORF) of the S gene cloned into pBK-CMV[smallS]. Dots indicate similarity to wild type. Bases where letter is displayed indicate dissimilarity to wild type.

A2.3 (overleaf) – Predicted aa sequence (in the *pol* ORF) of the S gene cloned into pBK-CMV[smallS]. Dots indicate similarity to wild type. Bases where letter is displayed indicate dissimilarity to wild type.

APPENDIX A3

PUBLICATIONS AND CONFERENCE SUBMISSIONS ARISING FROM THIS WORK